Investigation of Pharmacological Anti-diabetic Effect on Selected Traditional Chinese Herbs

By

LAM Fung Chun
B. Sc. (Hons.)
The Chinese University of Hong Kong

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Abstract

Diabetes mellitus (DM) is a metabolic disorder in glucose homeostasis affecting over 150 million people worldwide. Over 90% of the cases are classified as type 2 DM, which is caused by partial insulin deficiency and insulin resistance. Many traditional Chinese herbs and formulae have been used for the treatment of diabetes for a long time, but most of their pharmacological effects have not been scientifically studied.

Fourteen herbs were selected from several traditional Chinese herbal formulae used for diabetes treatment. In addition, previous *in vitro* studies indicated the potent anti-diabetic effect of Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis *in vitro*. Further fractionation has been processed to isolate the active compound(s) in Cortex Moutan. The potential anti-diabetic effects in all the above herbs and compounds were investigated using systematic approaches including various *in vitro* and *in vivo* models.

Four *in vitro* screening systems were employed for studying the effect of traditional Chinese herbs: (1) Gluconeogenesis in H4IIE hepatoma cells; (2) Glucose absorption in brush border membrane vesicles (BBMV); and (3) Glucose uptake in 3T3-L1 adipocytes and (4) Glucose uptake in Hs68 skin fibroblasts. Neonatal STZ-diabetic Wistar rat model was used as *in vivo* model to confirm the results from *in vitro* studies.

From the results, Cortex Phellodendri, Radix Ophiopogonis and Rhizoma Coptidis showed the most significant anti-diabetic effects among the 4 *in vitro* assays. However, none of the three herbs show significant hypoglycaemic and anti-hyperglycaemic effect in diabetic rat model. Cortex Moutan, Rhizoma Smilacis

Chinensis and Rhizoma Alismatis mixture in ratio 1:1:1 also did not exert any synergistic anti-hyperglycaemic effect. From the result of BBMV assay, it revealed that both hexane and dichloromethane extract of Cortex Moutan had very potent inhibitory effect on intestinal glucose absorption. Upon further investigations, five pure compounds (CM-D1 to 5) were purified from dichloromethane extract (CM-D). Using mass spectrometry and nuclear magnetic resonance techniques, the structures were elucidated, and they were identified as 2,5-dihydroxy-4-methylacetophenone (CM-D3), 2,5-dihydroxy-4-methoxyacetophenone (CM-D4) and 3-hydroxy-4-methoxyacetophenone (CM-D5). CM-D1 was identified as paeonol by another student previously. These four compounds, together with acetovanillone (which is also a compound found in Cortex Moutan), they showed significant suppression of glucose absorption in BBMV model. Sulfonation of paeonol (sodium paeonol sulfonate) showed no suppression of glucose absorption in BBMV model. CM-D1 and CM-D3 (both had very potent effects in BBMV assay) and acetovanillone were studied *in vivo*. Both 200 and 400 mg/kg of paeonol showed significant anti-hyperglycaemic effect in STZ-diabetic rat model, but the activity was not as strong as metformin (200 mg/kg); on the other hand, synthesized CM-D3 and acetovanillone did not show any effect on improving oral glucose tolerance.

Further studies should be done on studying the other mode of action in type 2 DM, exploring the structure-activity relationship of the compound purified from CM-D in BBMV model and investigating synergistic effect on the herbs in order to make a new herbal formula on treating type 2 DM.

Abstract in Chinese

摘要

糖尿病是一種醣代謝疾病，它影響著全球超過一億五千萬人，而超過百分之九十的個案是屬於因缺乏胰島素分泌及胰島素抵抗所引致的二型糖尿病 。從很久以前開始，很多單味中草藥及方劑已被用作治療糖尿病，但是它們的藥物機理並未有科學化的研究。

在本研究中，十四種中草藥從數個用於治療糖尿病的方劑中挑選出來。另根據同組研究員的試管實驗模型結果顯示，牡丹皮、菝葜和澤瀉具有潛在抗糖尿病的效果，而且牡丹皮中部分活性成分用分餾法分離出來。本研究會利用不同的試管實驗模型及動物模型去有系統地研究以上單味中草藥及化合物的抗糖尿效用。

利用四種不同的試管實驗模型我們可以研究中草藥抗糖尿的機制，包括：（１）Ｈ４ＩＩＥ大鼠肝癌細胞的動物澱粉新生模型；（２）刷狀緣膜囊泡的葡萄糖吸收模型；（３）３Ｔ３－Ｌ１小鼠脂肪細胞的葡萄糖吸取模型及（４）HS６８人類皮膚纖維組織母細胞的葡萄糖吸取模型。而鏈脲佐菌素所引致的二型糖尿病初生大鼠動物模型則用作確定試管實驗模型的結果。

實驗結果顯示，黃柏、麥冬和黃連在四種不同的試管實驗模型中表現出顯著的抗糖尿效果。但我們的動物實驗結果卻發現這三種單味藥均沒有降血糖或抗高血糖的效果。牡丹皮，菝葜和澤瀉在１：１：１比例下亦沒有抗高血糖的協用作用。在刷狀緣膜囊泡實驗中發現牡丹皮環己烷及二氯甲烷提取物能有效抑制小腸吸收葡萄糖。經過質譜及核磁共振技術分析，我們證實了它們是牡丹皮的一些已知成分包括２，５－二烴基－４－甲基苯乙酮，（ＣＭ－Ｄ３）、

２，５－二烴基－４－甲氧基苯乙酮（ＣＭ－Ｄ４）及３－氫基－４－甲氧基苯乙酮，（ＣＭ－Ｄ５）。ＣＭ－Ｄ１則已被另一位研究員確定爲丹皮酚。以上四個化合物再加上另一個同樣是牡丹皮活性成分的夾竹桃麻素均有顯著抑制小腸吸收葡萄糖吸收。磺化丹皮酚鈉則沒有抑制小腸吸收葡萄糖吸收之作用。由於ＣＭ－Ｄ１和ＣＭ－Ｄ３同樣有十分顯著抑制小腸吸收葡萄糖吸收之功效，它們連同夾竹桃麻素會被用在動物模特兒測試。結果發現每公斤體重二百和四百毫克的丹皮酚有明顯的抗高血糖效果，但活性不及西藥每公斤體重二百和四百毫克的二甲雙胍；另一方面，化學合成的ＣＭ－Ｄ３和夾竹桃麻素則沒有明顯改善口服葡萄糖耐量。

未來研究方向包括探究其他與二型糖尿病有關機理，探索各牡丹皮提取物的結構與刷纖緣膜囊泡的葡萄糖吸收的關係，以及研究其他中草藥的協同作用，以研製出新的治療二型糖尿病方劑。

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List of Abbreviations

^{1}H-NMR	Proton nuclear magnetic resonance
2-DOG	2-Deoxy-D-glucose
^{3}H	Tritium
ADA	American Dibetes Association
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BBMV	Brush border membrane vesicles
BCA	Bicinchoninic acid
Ca^{2+}	Calcium ion
Camp	Adenosine 3',5'-cyclic monophosphate
$CDCl_3$	Deuterium chloroform
cDNA	Compementry deoxyribose nucleic acid
$CHCl_3$	Chloroform
CH_2Cl_2	Dichloromethane
CH_3^+	Carbonium ion
CO_2	Carbon dioxide
$CuSO_4 \cdot 5H_2O$	Copper(II) sulphate pentahydrate
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribose nucleic acid

DNTP	Deoxynucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
EtBr	Ethidium bromide
EtOAc	Ethyl acetate
EtOH	Ethanol
F-1,6-BPase	Fructose 1,6-bisphasphatase
FBS	Fetal bovine serum
FPG	Fasting plasma glucose
G-6-P	Glucose-6-phosphate
G-6-Pase	Glucose-6-phosphatase
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter
GTT	Glucose tolerance test
H_2O	Water
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulphonic acid
HLA	Human leukocyte antigens
HPLC	High performance liquid chromatography
INS	Insulin
i.p.	Intraperitoneal
IBMX	3-Isobutyl-1-methylxanthine
IDDM	Insulin-dependent diabetes mellitus

IFG	Impaired fasting glycaemia
K^+	Potassium ion
KCl	Potassium chloride
KH_2PO_4	Monobasic potassium phosphate
MeOH	Methanol
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MHC	Major Histocompatibility Complex
mRNA	Massager ribose nucleic acid
MS	Mass spectrometry
Na^+	Sodium ion
Na_2HPO_4	Dibasic sodium phosphate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NaSCN	Sodium thiocyanate
NIDDM	Non-insulin-dependent diabetes mellitus
NO	Nitric oxide
OD	Optical distance
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
pCPT-cAMP	8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate
PCR	Polymerase chain reaction

PEPCK	Phosphoenolpyruvate carboxykinase
PPAR-γ	Peroxisome proliferators-activated receptor-gamma
RNA	Ribose nucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SEM	Standard error of the mean
SGLT1	Sodium ion/D-glucose cotransporter 1
STZ	Streptozotocin
TCM	Traditional Chinese Medicine
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
TZD	Thiazolidinediones
UV	Ultraviolet light
WHO	World Health Organization

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Publication

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Chapter 1 Introduction

1.1 Epidemiology of Diabetes Mellitus

Diabetes Mellitus (DM) or diabetes is a chronic and progressive metabolic disorder of glucose homeostasis which affects more than 150 million people in all age groups (International Diabetes Federation, 2000). The global figure of people with diabetes is rising, with at least 171 million people worldwide have DM in 2000, and the number is likely to be doubled or more by 2030 (World Health Organization, 2004a).

Prevalence of DM in developed and developing countries was 6.2% and 3.5% respectively in year 2000 (King *et al.*, 1998). DM caused 1.7% (around 988 thousands) of death worldwide in 2004, with mortality higher than stomach cancer, and it is ranked the second cancer killer (World Health Organization, 2004b). The majority of diabetic patients have type 2 DM; only 5 to 10% are suffering from type 1 DM (Koda-Kimble and Carlisle, 2001).

Following India, China has the second largest diabetic population in the world. The adult diabetic population in China was 20.8 million in 2000, which will increase to 42.3 million by 2030 (Wild *et al.*, 2004). In Hong Kong, diabetes is the eighth leading cause to death, accounting for 2% of death in 2001 (Department of Health, 2003).

1.2 Definition of Diabetes Mellitus

"Diabetes" and "Mellitus" are two words describing two well-known features of diabetic patients. The word "Diabetes" refers to excessive urine excretion (polyuria), while "Mellitus" means the urine is "honeyed" (glycosuria).

According to the report of World Health Organization (WHO) published in 1999,

the latest definition of diabetes mellitus is "a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both" (World Health Organization, 1999).

1.3 Glucose Homeostasis and Diabetes Mellitus

Glucose is the major energy source of human body and transported to the whole body through the circulation system. Pancreas is the organ responsible for the regulation of blood glucose, by the action of two hormones: insulin and glucagon. The pancreas secretes insulin when blood glucose level is high, normally post-prandial, to inhibit the endogenous glucose production and stimulate the glucose utilization and storage. As the blood glucose decreases, insulin release is inhibited and the pancreas releases glucagon, as the counter-regulatory hormone, to maintain the basal glucose level.

Insulin is the glucose lowering hormone secreted by β-cells in the islets of Langerhans of the pancreas. The islets of Langerhans are clusters of endocrine tissue scattered throughout the pancreas (Bonner-Weir, 1991). In addition to β-cells which are the most common cell type, the islets also contain glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing PP-cells. Insulin is originated from proinsulin, a single-chain protein. A connecting peptide (C peptide) is cleaved from proinsulin to form insulin (Fig. 1.1). Insulin is a 51 amino acid polypeptide with two peptide chains connected by two disulphide bonds (Rhodes, 2004; Rhodes, 2004).

Along with basal insulin release, β-cells store insulin in granules. Increase of blood glucose level causes depolarization of β-cells. This results in influx of calcium ion and exocytosis of insulin containing granules, called the first phase of insulin release.

Glucose also activates the protein kinase C, which is responsible for the sustained insulin release, called the second phase (Fig. 1.2) (Straub and Sharp, 2004).

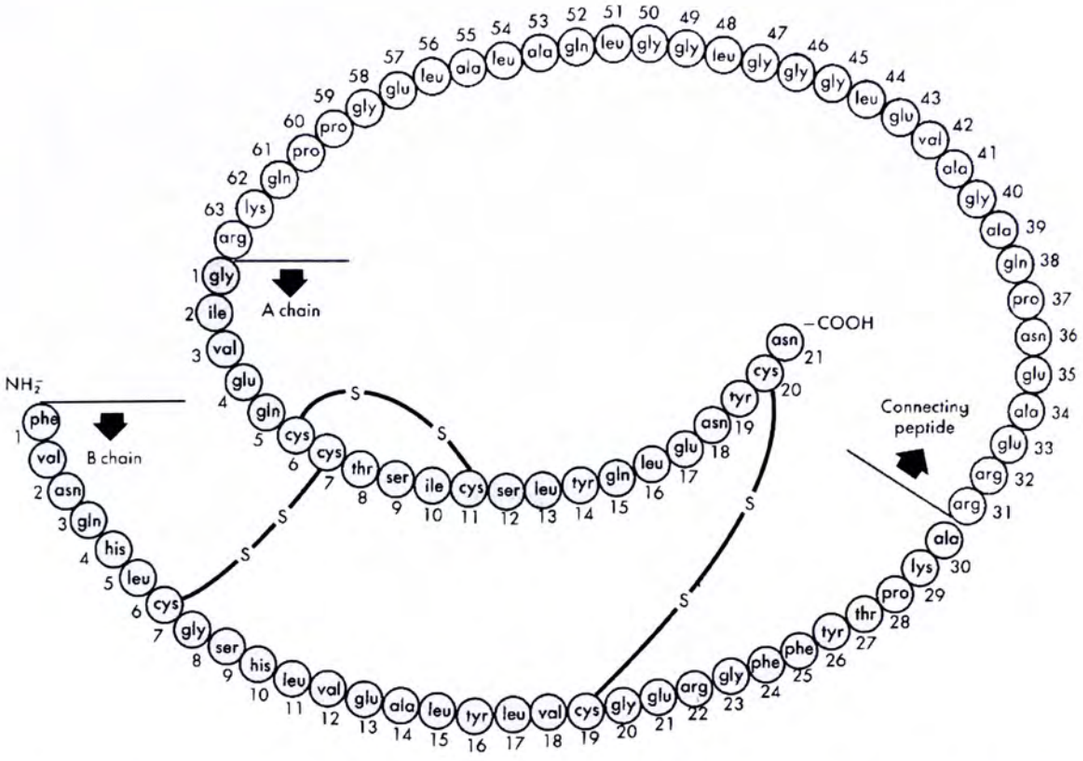


Fig. 1.1 Structure of Porcine Proinsulin. Insulin is originated from preproinsulin and proinsulin. After cleavage of connecting peptide (C peptide; white area) from proinsulin, two peptide chains A chain and B chain (grey area) connected by disulphide bonds and form 51 amino acid polypeptide insulin. Insulin is a 51 amino acid polypeptide with two peptide chains (Genuth, 1998).

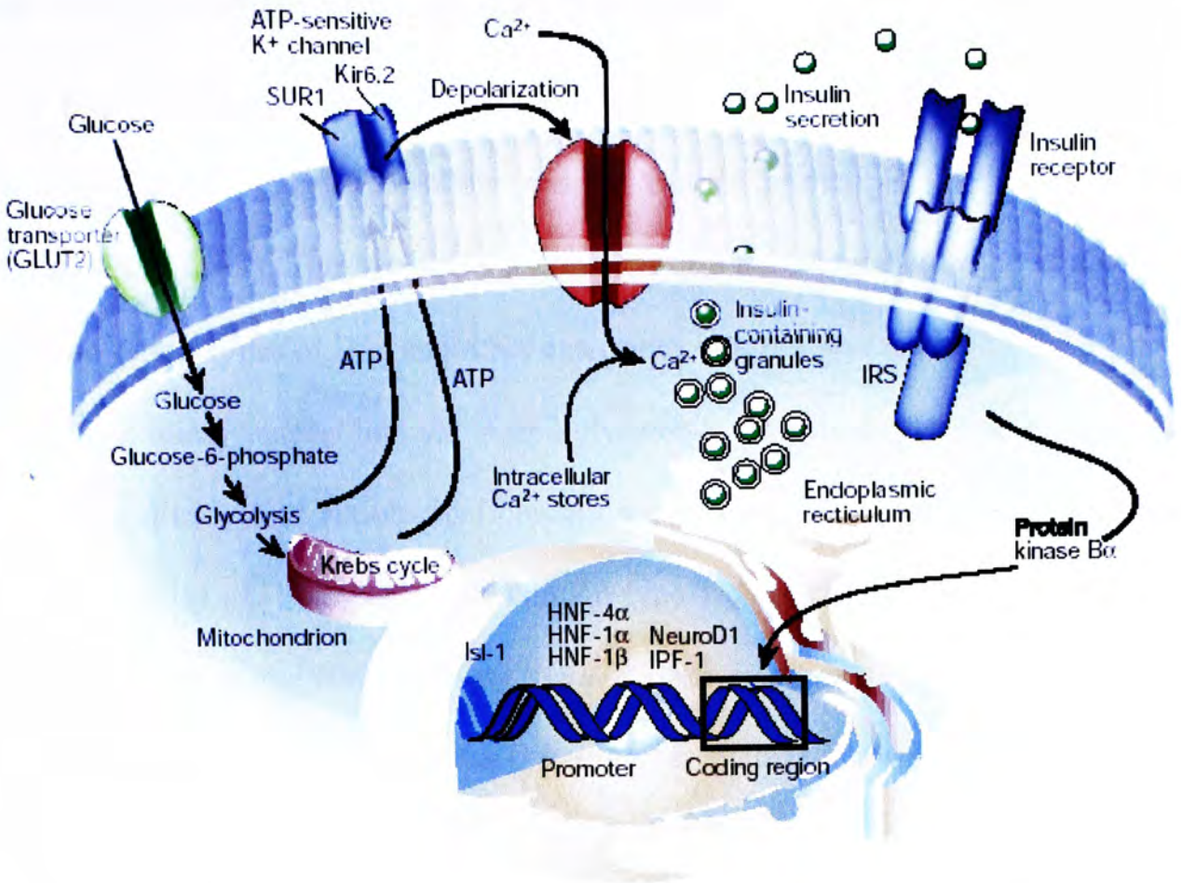


Fig. 1.2 Model of insulin secreting pancreatic β-cell. Glucose is transported into pancreatic β-cell through glucose transporter 2 (GLUT2). Glucose is catalyzed to form gluocose-6-phosphate by glucokinase with a phosphate (from ATP) added. The generation of ATP by glycolysis and the Kerbs cycle leads to closure of the ATP-sensitive K^+ channels, which leads to depolarization of plasma membrane and extracellular calcium influx. This influxed calcium changed into intracellular storage. The insulin-containing secretory granules then fuse with plasma membrane and insulin is released into circulation (Bell and Polonsky, 2001).

1.4 Classification of Diabetes Mellitus

The world Health Organization (WHO) has revised its 'Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications' in 1999 (World Health Organization, 1999). The revised classification criteria contain both the clinical stages and aetiological types of DM and other categories of hyperglycaemia.

The clinical stages include normoglycaemia, impaired glucose regulation and diabetes mellitus. And aetiological classification refers defects, disorders or processes that lead to DM. Aetiological types contain type 1 diabetes mellitus (previously called insulin dependent diabetes mellitus), type 2 diabetes mellitus (previously called non-insulin dependent diabetes mellitus), gestational diabetes mellitus and other specific types.

1.4.1 Type 1 Diabetes Mellitus

Type 1 DM is an autoimmune disease resulting from specific destruction of insulin-producing β-cells of islet of Langerhans of the pancreas, and is characterised by absolute insulin deficiency (Tisch and McDevitt, 1996). It affects 0.5% of population in developing countries and increasing incidence (Mathis *et al.*, 2001). There are 2 phases of Type 1 DM, the first is insulitis in which a mixed population of leukocytes invades the islets; while the second is diabetes in which most β-cells have been killed and no sufficient insulin can be produced for regulating circulating blood glucose level, and it leads to hyperglycaemia.

There is an increasing risk of developing type 1 DM in first-degree relatives, especially on monozygotic twins. By studying the extensive sequencing of Major Histocompatibility Complex (MHC) class II alleles in man, it revealed that type 1 DM is closely interplayed with two major isotypes of MHC class II molecules HLA-DR and

HLA-DQ (Wicker *et al.*, 1995), and also the presence of antibodies against islet cells or glutamic acid decarboxylase (Koda-Kimble and Carlisle, 2001).

Type 1 diabetes usually presents in children and adolescents, but it also occurs in elderly. Individuals who have type 1 diabetes depend on insulin therapy for survival (Ergun-Longmire *et al.*, 2004). However, for the type 1 idopathic diabetic patients, they develop ketoacidosis without autoimmune destruction of the pancreas. Such traits are of unknown aetiologies and strongly inherited (Tiberti *et al.*, 2000).

1.4.2 Type 2 Diabetes Mellitus

Type 2 DM is a heterogeneous disorder characterised by diminished liver, muscle, and adipose tissue sensitivity to insulin (insulin resistance) and impaired pancreatic β-cell function (World Health Organization, 1999). Defects in insulin action can lead to impaired insulin secretion and vice versa, while impaired insulin secretion and hyperglycaemia can reduce insulin action. Also, when β-cells lose the ability to respond to glucose elevation after meal, insulin secretion is impaired and blood glucose level becomes out of control, then leading to Type 2 DM. The elevated blood glucose level may exist for many years before diagnosis, and result in damage of various target tissues before clinical symptoms. Genetic factor, obesity, sedentary lifestyle and aging are the believed risk factors of insulin resistance and type 2 DM (World Health Organization, 1999).

Insulin resistance is defined as decreasing sensitivity of body tissue towards insulin, and it needs greater amount of insulin to elicit a normal response. Hyperinsulinaemia is a common feature in patients with an early insulin resistance. Insulin resistance is now known to be present at early stage of type 2 DM (American Diabetes Association, 1998).

The symptoms of type 2 DM include fatigue, polyuria and polydipsia, but these are

usually mild and with gradual onset. Unexpected weight loss and ketosis are less common in type 2 diabetic patients. Individuals with type 2 diabetes do not need exogenous insulin for survival, although it may be required in patients with serious symptoms (Koda-Kimble and Carlisle, 2001).

1.4.3 Gestational Diabetes Mellitus

Hyperglycaemia during pregnancy is known as gestational diabetes mellitus (GDM). Women with GDM are at high risk of developing diabetes when they are not pregnant. Also, women who have GDM would have higher chances to develop type 2 diabetes afterward (Buchanan *et al.*, 1998; Xiang *et al.*, 1999).

1.4.4 Other specific types

Only 1 to 2% of cases belong to this type of diabetes (Harris, 2004). Here is the list of different reasons resulting in diabetes:

i) Genetic defects of β-cell function

ii) Genetic defects in insulin action

iii) Diseases of the exocrine pancreas

iv) Endocrinopathies

v) Drug or chemical induced

vi) Infections

vii) Uncommon forms of immune- mediated diabetes

viii) Other genetic syndromes

(World Health Organization, 1999)

1.5 Diagnostic Criteria of Diabetes Mellitus

American Diabetes Association (ADA) revised the diagnostic criteria on diabetes mellitus in 2002 (refer to Table 1.1) (American Diabetes Association, 2004). It is based on the measure of hyperglycaemia by three criteria: basal plasma glucose level, fasting plasma glucose (FPG) and 2-hour post glucose load plasma glucose (oral glucose tolerance). Fasting plasma glucose level is the most commonly used diagnostic criteria for diabetes mellitus as it is easy to use, accepted by patients and with low costs. Fasting plasma glucose lower than 5.5 mM is considered as normal, while 7.0mM or above is considered as diabetes mellitus. Impaired fasting glucose (IFG) is plasma glucose level in between the two values. Oral glucose tolerance test (OGTT) is recommended if the result of FPG could not give the prominent result for diagnosis. The test is administrated in the morning after at least three days of unrestricted diet (greater then 150g of carbohydrate daily) and usual physical activity, followed by an overnight fasting of 8 to 14 hours. After collecting the fasting blood sample, the subject should drink 75g of anhydrous glucose in 250 to 300ml water within five minutes. Blood sample is collected after 2 hours, and plasma glucose level is determined (World Health Organization, 1999). Normal people should have a value smaller than 7.8mM, while diabetes patients have a result of 11.1mM or above. IGT is diagnosed if the OGTT result is 7.8mM or above but smaller than 11.1mM.

People without any obvious symptoms but shows hyperglycaemia in at least two test results is claimed to be diabetic patient. Moreover, hyperglycaemia detected under stress such as infection could not be regarded as diagnostic of DM.

	Basal plasma glucose	Fasting plasma glucose (FPG)	Oral glucose tolerance test (2hr post glucose load)
Normal	/	≤100mg/dl (≤5.5mM)	<140 mg/dl (<7.7mM)
Impaired glucose tolerance	/	100-125 mg/dl (5.6 – 6.9mM)	140-199 mg/dl (7.7 – 11.1mM)
Diabetes	≥200 mg/dl (≥11.1mM)	≥126 mg/dl (≥7.0mM)	≥200 mg/dl (≥11.1mM)

Table 1.1 Diagnostic criteria of diabetes mellitus. Basal plasma glucose is defined as the plasma glucose level at any time of day. Fasting is defined as no caloric intake for at least 8 hours; Oral glucose tolerance test should be performed by using a glucose load containing the equivalent of 75g glucose dissolved in 250-300ml water (American Diabetes Association, 2004).

1.6 Complications of Diabetes Mellitus

Complications could occur when the blood glucose level of the patients is not maintained at physiological range. The major risk factors for the marcovascular and microvascular complications of diabetes are hypertension, hyperlipidemia, hyperglycaemia, lack of exercise and smoking. Most of these risk factors are more prevalent in the type 2 diabetic population and synergistically to promote vascular disease.

Here are the common diabetic complications:

Cardiovascular complications - It includes coronary heart disease and strokes, are the most common cause of morbidity and mortality in diabetes. Its occurrence is positively associated with duration of diabetes (Resnick *et al.*, 2004). Hyperglycaemia promotes the formation of advanced glycosylaton (glycation) products, which cross-link with collagen, causing arterial stiffness. Atherogenesis would appear as increased levels of low-density lipoprotein (LDL) cholesterol (Bate and Jerums, 2003).

Retinopathy - Up to 12% of type 1 and 5% of type 2 diabetic patients become blind after 30 years of diabetes because of diabetes retinopathy or cataract (Chew, 2004; Watkins *et al.*, 2003). It is due to hemorrhages and angiogenesis in the retina leading to damaged vision (Ewing *et al.*, 1998).

Nephropathy - Diabetes could damage the glomeruli of the kidney and decrease the glomerular filtration rate, leading to proteinuria and hyperuricaemia (National Institute of Diabetes and Digestive and Kidney Diseases, 1995). It affects 20–40% of the diabetic patients, for some case kidney transplantation are needed (Ruggenenti and Remuzzi, 2000).

Neuropathy - It has a wide variety of manifestation in diabetic patients, and these neuropathies are among the most common and perplexing complications of diabetes

mellitus (American Diabetes Association, 1998). More than half of diabetic patients develop neuropathy (Feldman, 2003) and it is clear that impaired blood flow and endoneurial hypoxia are the major causes (Cameron *et al.*, 2001). Peripheral and autonomic nerve dysfunction would cause a painful syndrome, featuring numbness, diarrhea, postural hypotension, respiratory arrests, sweating and erectile dysfunction (Watkins *et al.*, 2003).

Foot ulcer - For diabetes patients, foot ulcer and amputation are the major cause of morbidity and disability (Frykberg *et al.*, 1998). Foot lesions are commonly found on the patient's foot, and about 15% of the diabetic patients will develop foot ulcer (Edmonds and Foster, 2004). Polyneuropathy, peripheral vascular disease, superimposed infection or a combination of these complications would cause numbness and ischaemia of foot, and gradually develop into foot ulcer (American Diabetes Association, 1998). Unhealing ulceration usually leads to amputation in the worst cases (Watkins *et al.*, 2003). Current therapy is mainly performed by applying antibiotics and intensive wound care (Edmonds and Foster, 2004; Mason *et al.*, 1999).

1.7 Pharmacological Treatment of Diabetes

The management goals of diabetes mellitus are mainly to achieve normal glyceamic control, and it is the key to prevent long-term complications, so as to improve the quality of life of the patients. There are three main kinds of treatments for different types of diabetes mellitus: dietary modification, regular physical activity and pharmacological approach with either oral hypoglycaemic agents or insulin.

1.7.1 Treatment for type 1 diabetes mellitus

Exogenous insulin is the only option currently available as replacement therapy to

compensate for the lack of endogenous insulin in type 1 DM. Insulin could decrease the blood glucose level by promoting glucose uptake from blood into target cells (adipocytes, hepatocytes and also skeletal muscle cells) which is responsible for carbohydrates, lipid and protein metabolism. At the cellular level, insulin could stimulate glycogen synthesis, lipid synthesis and glucose oxidation, while it could also inhibit glycogenolysis, lipolysis and gluconeogenesis.

Exogenous insulin is absorbed through the circulation system after subcutaneous injection, and the patients need to inject insulin around 4 times per day and 30 minutes before meal, in order to match the physiological peak of insulin secretion of normal people (American Diabetes Association, 1998).

There are many adverse effects of insulin injection, such as hypoglycaemia (due to hyperinsulinemia), lipodystrophy (cause allergic reaction on injection site), defective counter-regulatory hormonal response and weight gain (Koda-Kimble and Carlisle, 2001). Hence, various insulin analogues were produced (insulin lispro, insulin aspart, insulin glargine and insulin detemir) to overcome the hypoglycaemic effect by altering the onset and duration period (Dunn and Plosker, 2002; Gillies *et al.*, 2000; Goldman-Levine and Lee, 2005; Simpson and Spencer, 1999).

Pancreas or pancreatic islets transplantation is another method to overcome long-term insulin injection and also daily inspection on blood glucose for type 1 diabetic patients (Burke *et al.*, 2004). However, the success rate of transplantation is relatively low as chronic rejections and recurrence of autoimmunity may occur (Lohmann *et al.*, 2002).

1.7.2 Treatment for Type 2 diabetes mellitus

Type 2 diabetic patients usually suffer from insulin resistance or insulin deficiency

or both. Hence insulin therapy could not be used in this case. Dietary control and exercise may help to control blood glucose for each meal and exercise could improve the state of insulin resistance (increasing insulin sensitivity) of muscle cells and adipocytes. By means of these methods, body weight could be controlled and increase glucose uptake by muscle cells and adipocytes.

However, oral anti-diabetic drugs could be applied if non-medication treatments do not work. These drugs act on the target tissues which are involved in glucose metabolism by different modes of action: i) stimulating insulin release; ii) inhibiting hepatic gluconeogenesis; iii) inhibiting glucose absorption from intestine; and iv) improving insulin sensitivity of peripheral tissues. Insulin may be used when blood glucose is not under control even if oral medications are applied.

There are five classes of anti-diabetic drugs nowadays, namely sulfonylureas, meglitinides, biguanides, thiazolidinediones and α-glucosidase inhibitors.

1.7.2.1 Sulfonylureas

Sulfonylureas stimulate the pancreatic β-cells to release more insulin (Zimmerman, 1997). Sulfonylureas could bind onto specific sulphonylurea receptors on the pancreatic β-cells. Such binding results in inhibition of ATP-dependent K^+ channel and cause depolarization of the plasma membrane by calcium ion (Ca^{2+}) influx due to intracellular K^+ increases. Finally, insulin is released from insulin-containing granules (Chehade and Mooradian, 2000).

The most common side effects of sulfonylureas are hypoglycaemia (Bayraktar *et al.*, 1996; Lam *et al.*, 1998) and weight gain (Kabadi and Kabadi, 2003). The drugs (except glyburide) are also not suitable for pregnant or breast-feeding women because they can cross the placenta and can be excreted into breast milk (Koda-Kimble and Carlisle, 2001).

Some examples of sulfonylureas are glimepiride, glyburide, chorpropamide, acetohexamide, glipizide, glyburide, tolbutamide and tolazamide (American Diabetes Association, 1998).

1.7.1.2 Meglitinides

Meglitinidies also stimulate pancreatic β-cells to release insulin. Its action is faster then sulfonylureas as it has rapid onset and short duration, hence it should be administrated shortly after the meal (Fuhlendorff *et al.*, 1998). It has potent oral hypoglycaemic activity and is structurally different from the sulphonylureas, although it stimulates Ca^{2+} influx by binding to the different receptor sites compared with suphonylurea on pancreatic β-cells and closing ATP-dependent K^{+} channels (Adis International Limited, 2004; Dunn and Faulds, 2000).

It is important in reducing long-term cardiovascular complications of diabetes. Its side-effects are hypoglycaemia and weight gain, but not as serious as sulfonylureas do (Plosker and Figgitt, 2004). Examples of meglitinides are repaglinide and nateglinide (American Diabetes Association, 1998) .

1.7.1.3 Biguanides

Biguanides were developed from a traditional plant remedy, *Galega officinalis* (French Lilac, goat's rue). Guanidine is the plant extract proved to have mild antidiabetic activity in 1918. But due to its toxicity and pathological effects, it is not suitable for clinical use (Keeler *et al.*, 1992).

Phenformin and metformin were then developed, but nowadays only metformin was used as because phenformin showed lethal side-effect and lactic acidosis (Bailey, 1992). Metformin reduces blood glucose by means of enhancing the glucose uptake by

skeletal muscle cells and adipocytes through glucose transporter type 4 (GLUT4). It also exerts effects on hepatocytes to inhibit the hepatic gluconeogenesis, so as to lower the fasting blood glucose level. Metformin increases insulin sensitivity but only with the presence of insulin in the patients as well (Bailey, 1992; Bailey and Turner, 1996).

However, metformin would cause lactic acidosis, hence it could not be used on patients who has renal impairment (Bailey and Turner, 1996).

1.7.1.4 Thazolidinediones

Thiazolidinediones (TZDs) is a new class of antidiabtic drugs which known as peroxisome proliferators-activated receptor-gamma (PPAR-γ) agonists (Krentz *et al.*, 2000). PPAR-γ is a transcription factor that is found mainly in adipose tissues (Vidal-Puig *et al.*, 1997). It lowers the blood glucose level by increasing insulin sensitivity in skeletal muscle and adipocytes (Kim *et al.*, 2004a), thus insulin-stimulated signal transduction is enhanced (Miyazaki *et al.*, 2003).

Ciglitazone was the first TZD derivatives, and then followed by troglitazone, pioglitazone, rosglitazone and englitazone (Saltiel and Olefsky, 1996). Troglitazone was the first discovered TZD but due to hepatotoxic effect, it was soon stopped in the market (Graham *et al.*, 2003).

The major adverse effects of TZDs are weight gain, hepatocytotoxity, edema and anemia (Lebovitz, 2002). Recently TDZs was also discovered to cause congestive heart failure (Cheng and Fantus, 2004).

1.7.1.5 α-Glucosidase inhibitor

α-Glucosidase is an enzyme which locates in brush border of the small intestine and is responsible for breaking down non-absorbable carbohydrates into absorbable

monosaccharides. α-Glucosidase inhibitors are reversible competitive inhibitors of this enzyme. Acarbose is one of the most commonly used α-glucosidase inhibitors (Bischoff, 1994).

Acarbose mainly exerts its antidiabetic function by reversibly inhibit α-glucosidase by binding to the active site of the enzyme, and preventing its cleavage of substrate (complex carbohydrates) (Salvatore and Giugliano, 1996). Thus, it could slow down the absorption of carbohydrates from intestine of the patients, and lower the postprandial blood glucose level. But acarbose has no effect on monosaccharides which do not need to be cleavaged by α-glucosidase (Yee and Fong, 1996; Bischoff, 1994).

Gastrointestinal disturbance such as flatulence, abdominal distension and diarrhea may occur because of the presence of undigested complex carbohydrates in the colon if the dosage of the drug is low (Chiasson *et al.*, 1994). Moreover, using acarbose at a dose larger than 300 mg/day could cause liver damage (Coniff *et al.*, 1995).

1.8 Diabetes and Traditional Chinese Medicine

In Chinese medical pathology, diabetes mellitus is defined as "Xiao Ke Zhang" (消渴症). Patients who suffered from "Xiao Ke Zhang" would have thirsty feeling, sweet urine and weight loss. This is due to the deficiency of *Yin* (陰) and heat inside stomach, kidney and lung, leading to water loss. Traditional Chinese medical expert book "Huang Di Nei Jing" (黃帝內經) claimed that diabetes is resulted from long-term intake of fatty and sweet food. The treatment of "Xiao Ke Zhang" is mainly nourish yin, restoring water content inside body by clearing heat, and also tonifying *Qi* (氣). Many traditional Chinese medicines (TCMs) have been used for treatment of diabetes mellitus. A survey

was done by my colleague Dr. David Lau among the traditional Chinese herbal formulae in treating diabetes mellitus (see Appendix 1).

1.9 Objective of this project

Type II diabetes mellitus is a chronic metabolic disorder with increasing prevalence. Many western oral medications have been developed for treatment, but most of them have side effects, and cause serious kidney failure with long-term application. Traditional Chinese herbs which have fewer side effects could be an alternative means for diabetes control.

Fourteen herbs were selected from the traditional Chinese herbal formulae (Table 1.2), according to their frequency of usage and also the dosage indicated in the list mentioned above (Appendix 1). Various *in vitro* models for intestinal glucose absorption, hepatic glucose production and cellular glucose uptake were used to screen the selected herbs for their potential anti-diabetic effects. Herbs showing promising effects *in vitro* were further investigated *in vivo* using diabetic rat model.

There were three herbs chosen from formula 1 "Tuo Du Sheng Ji Tang" (托毒生肌湯) and formula 2 "Qi Wei Di Huang Tang" (耆味地黃湯), namely Cortex Moutan (牡丹皮), Rhizoma Smilacis Chinensis (菝葜) and Rhizoma Alismatis (澤瀉), they all showed the most potent anti-diabetic effects in various *in vitro* assays done by another two students (Chan, 2004; Lau, 2004). Formula 1 and formula 2 were developed for treating diabetic foot ulcer by a Chinese herbalist in order to prevent amputation in type 2 DM patients (Wong *et al.*, 2001). The ingredients of both formulae are shown in table 1.3. As the chosen herbs showed potent anti-diabetic effects *in vitro*, the results were confirmed by *in vivo* model.

Furthermore, bioassay-guided fractionation of *Paeonia suffruticosa* (牡丹皮) was employed in order to isolate the active component(s) and the chemical structure(s) was elucidated.

Latin Name	Chinese Name
Paeonia lactiflora Pall.	白芍
Platyclatus orientalis (L.) Franco	柏子仁
Codonopsis pilosula (Franch.) Nannf.	黨參
Lophatherum gracile Brongn.	淡竹葉
Glycyrrhiza uralensis Fisch.	甘草
Pueraria lobata (Willd.) Ohwi	葛根
Cinnamomum cassia Presl	桂枝
Phellodendron amurense Rupr.	黃柏
Coptis chinensis Franch.	黃連
Ophiopogon japonicus (L.f.) Ker-Gawl.	麥冬
Trichosanthes kirilowii Maxim.	天花粉
Pseudostellaria heterophylla (Miq.) Pax ex Pax et Hoffm.	太子參
Ziziphus jujuba Mill. var. spinosa (Bunge) Hu ex H. F.Chou	棗仁
Anemarrhena asphodeloides Bge.	知母

Table 1.2 Fourteen herbs were selected according to survey in Appendix 1.

Formula 1		
Radix Astragali	黃耆	20g
Radix Polygoni Multiflori Preparata	制首烏	9g
Radix Rehmanniae	生地黃	9g
Radix Stephaniae Tetrandrae	漢防己	9g
Rhizoma Atractylodis Marcocephala	白朮	9g
Rhizoma Smilacis Chinensis	菝葜	9g

Formula 2		
Cortex Moutan	牡丹皮	6g
Fructus Corni	山茱萸	9g
Fructus Schisandrae Chinensis	五味子	6g
Poria	茯苓	6g
Radix Astragali	黃耆	20g
Radix Rehmanniae	生地黃	12g
Rhizoma Alismatis	澤瀉	6g
Rhizoma Dioscoreae	山藥	9g

Table 1.3 The herbal ingredients of Formula 1 and Formula 2.
(Wong *et al.*, 2001)

Chapter 2 Botanical, Preparation and Authentication of Traditional Chinese Herbs

2.1 Introduction

In this chapter, the background and properties of each selected herbs will be mentioned, also authentication of each herbs will be discussed.

As last chapter mentioned, the purpose of my project is to select some traditional Chinese herbs other then Formula 1 "Tuo Du Sheng Ji Tang"and Formula 2 "Qi Wei Di Huang Tang", then investigate the synergistic effect of the effective herbal extracts in treating diabetes mellitus.

The three herbs that showed significant effects in *in vitro* assays in Formula 1 and Formula 2 are chosen for further studies (Wong *et al.*, 2001); they are Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis. And for the other 14 herbs chosen from 12 traditional herbal formulae (Table 1.2); they were selected according to their frequency and dosage used.

2.2 Herbal Materials

In this part, the information of Chinese medicinal properties and some research findings regarding on the function of each traditional herb will be discussed.

Cortex Moutan (牡丹皮)

Cortex Moutan (Mudanpi) is the dried root bark of *Paeonia suffruticosa* of the family Ranunculaceae. It is used to remove *heat* from blood, to activate blood circulation and eliminate *blood stasis* (State Pharmacopoeia Commission, 2000). Recent studies found that polysaccharides isolated from Cortex Moutan possessed hypoglycaemic effect on alloxan-induced diabetic mice and streptozotocin (STZ, also known as streptozotocin) -induced diabetic rats (Wang *et al.*, 2001; Hong *et al.*, 2003)

Rhizoma Smilacis Chinensis (菝葜)

Rhizoma Smilacis Chinensis is the dried rhizome of *Smilax china* L. [*S. japonica*]. It is usually used to treat rheumatic disease, dysentery and upset stomach (State Pharmacopoeia Commission, 2000). It is also found to have diuretic action and promotional effect on blood circulation (Liu *et al.*, 2002). No report concerning the effects of this herb on diabetes has been published.

Rhizoma Alismatis (澤瀉)

Rhizoma Alismatis (Zexie) is the dried tuber of *Alisma orientalis* of the family Alismataceae. In TCM theory, its action is to cause diuresis, and to remove

damp-heat (State Pharmacopoeia Commission, 2000). Water-ethanol extract of Rhizoma Alismatis was found decreasing plasma glucose and triglycerides, and increasing plasma insulin of alloxan-induced and STZ-induced diabetic mice (Yang *et al.*, 2002a; Yang *et al.*, 2002b).

Radix Paeoniae Alba (白芍)

Radix Paeoniae Alba (Baishao) is the dried root of *Paeonia lactiflora* (Family Ranunculaceae). It is used to subdue hyperactivity of the liver and relieve pain, to nourish blood and regulate menstruation, and to check excessive perspiration (State Pharmacopoeia Commission, 2000).

Semen Platycladi (柏子仁)

Semen Paeoniae Alba (Baiziren) is the dried ripe kernel of *Platycladus orientalis* (L.) Franco (Family Cupressaceae). It is commonly used to calm the nerves, to check excessive peripiration, and to relax the bowels (State Pharmacopoeia Commission, 2000). No diabetes related research of this herb has been done.

Radix Codonopsis (黨參)

Radix Codonopsis (Dangshen) is the dried root of *Codonopsis pilosula* Franch. (Family Campanulaceae). According to TCM theory, it is used to reinforce *Qi* and invigorate the function of spleen and the lung. It is commonly used to treat diabetes caused by internal *heat* (State Pharmacopoeia Commission, 2000). No diabetes related research of this herb has been done.

Herba Lophatheri (淡竹葉)

Herba Lophatheri (Danzhuye) is the dried stem and leaf of *Lophatherum gracile* Brongn. (Family Gramineae). It is used to remove *heat*, ease the mind and cause diuresis (State Pharmacopoeia Commission, 2000). No diabetes related research of this herb has been done.

Radix Glycyrrhizae (甘草)

Radix Glycyrrhizae (Gancao; licorice) is the dried root and rhizome of *Glycyrrhiza uralensis* Fisch. (Family Leguminosae). It can reinforce the function of the spleen and the stomach marked by lassitude and weakness, cardiac palpitation and shortened of breath, cough with much phlegm, spasmodicsores. It is often used for reducing the toxic or drastic strength, arrhythmia (State Pharmacopoeia

Commission, 2000). The ethanolic extract of *Glycyrrhiza uralensis* (100 to 300 mg/kg) could drcrease blood glucose level in younger (6 wk old) and older (13 wk old) diabetic KK-Ay mice (Mae *et al.*, 2003). Also, Glycyrin, one of the main PPAR-γ ligands of licorice, significantly decreased the blood glucose levels of genetically diabetic KK-Ay mice (Kuroda *et al.*, 2003).

Radix Puerariae (葛根)

Radix Puerariae is the dried root of *Pueraria lobata* Willd. (Famiy Leguminosae). It can be used to relieve fever, to promote the production of body fluid, to facilitate eruption, and to arrest diarrhea. It is a well-known herb which commonly used to treat diabetes (State Pharmacopoeia Commission, 2000). Puerarin, an active component in Radix Puerariae, can increase the glucose utilization to lower plasma glucose in diabetic rats lacking insulin (Hsu *et al.*, 2003). *Pueraria lobata* also has many other applications, such as anti-oxidant, anti-tumor, healing cardiovascular disease etc.

Ramulus Cinnamorni (桂枝)

Ramulus Cinnamomi is the dried young branch of *Cinnamomum cassia* (Family Lauraceae). It is used to induce perpiration, to warm the channels and

stimulate menstrual discharge, to reinforce yang, to relieve palpitation, and to promote the decending of *qi* (State Pharmacopoeia Commission, 2000). A decrease in blood glucose levels was observed in a glucose tolerance test (GTT) in rat model after applying *Cinnamomum cassia* extract; furthermore, the elevation in plasma insulin was direct since a stimulatory *in vitro* effect of insulin released from INS-1 cells (an insulin secreting cell line) was observed (Verspohl *et al.*, 2005).

Cortex Phellodendri (黃柏)

Cortex Phellodendri is the dried root of *Phellodendron amurense* Rupr. (Family Rutaceae). It is used to remove *damp-heat*; quench *fire*, counteract toxicity, and relieve consumptive fever (State Pharmacopoeia Commission, 2000). No diabetes related research related to this herb has been done.

Rhizoma Coptidis (黃連)

Rhizoma Coptidis is the dried rhizome of *Coptis chinensis* Franch. (Family Ranunculaceae). It is used to remove *damp-heat*, quench *fire* and counteract toxicity (State Pharmacopoeia Commission, 2000). Crude extract of Rhizoma Coptidis could decrease the serum glucose level in normal mice. A study showed that berberine, the major component in Rhizoma Coptidis, had an anti-hypoglycemic effect in normal,

alloxan-induced, and spontaneous diabetic KK mice. It could also suppress the hyperglycemic effect induced by intraperitoneal injection (i.p.) of glucose in normal mice, reduced the cholesterol level of the mice and also inhibit the aggregation of rabbit platelet *in vitro* (Chen and Xie, 1986). A recent study also showed that it could cause apoptosis on human colorectal cancer cells *in vitro* (Chen and Xie, 1986; Kim *et al.*, 2004b).

Radix Ophiopogonis (麥冬)

Radix Ophiopogonis is the dried root tuber of *Ophiopogon japonicus* Thunb. (Family Liaceae). It is used to nourish *yin* and promote the production of body fluid, to moisten the lung, and to ease the mind. It can be used to treat diabetes caused by internal *heat* (State Pharmacopoeia Commission, 2000). A study showed that it had a hypoglycaemic effect on streptozotocin-induced diabetic mice (Kako *et al.*, 1995). Polysaccharides of Radix Ophiogonis had an anti-hyperglyceamic activity on alloxan-induced diabetic mice (Zhang and Wang, 1993).

Radix Trichosanthis (天花粉)

Radix Trichosanthis is the dried root of *Trichosanthes kirilowii* Maxim (Family Cucurbitaceae). It is to remove *heat*, to promote the production of body fluid,

and to facilitate the drainage of pus and subsidence of swelling. It can be used to treat diabetes caused by internal *heat* (State Pharmacopoeia Commission, 2000). Study proved that five glycans trichosans A, B, C, D and E showed hypoglycemic actions in normal mice. The main glycan, trichosan A, also exhibited activity in alloxan-induced hyperglycemic mice (Hikino *et al.*, 1989).

Radix Pseudostellariae (太子參)

Radix Pseudostellariae is the dried root tuber of *Pseudostellaria heterophylla* Miq. (Family Caryophyllaceae). It is to replenish *qi* and invigorate the spleen function, and to promote fluid secretion and moisten the lung (State Pharmacopoeia Commission, 2000). *Pseudostellaria heterophylla* exhibits both immunomodulatory and anti-tumor activities(Wong *et al.*, 1994), but no related studies is done on its anti-diabetic effect.

Rhizoma Anemarrhenae (知母)

Rhizoma Anemarrhenae is the dried rhizome of *Anemarrhena asphodeloides* Bge. (Family Liliaceae). It removes heat and quenches fire, and promotes the production of body fluid and relieves the dryness syndrome. It can be used to treat diabetes due to internal *heat* (State Pharmacopoeia Commission, 2000). Study

showed the water extract of Radix Ophiopogonis (90mg/kg) could reduce the blood glucose level after oral administration (Miura *et al.*, 2001b), also its active component mangiferin and mangiferin-7-O-β-D-glucoside exerted an anti-diabetic effect by increasing insulin sensitivity (Miura *et al.*, 2001a; Miura *et al.*, 2001b).

Semen Ziziphi Spinosae (棗仁)

Semen Ziziphi Spinosae is the dried ripe seed of *Ziziphus jujuba* Mill. (Family Rhamnaceae). It is used to replenish the liver, to cause tranquilizations, to arrest excessive perspiration, and to promote the production of body fluid (State Pharmacopoeia Commission, 2000). No diabetes related research has been done using this herb.

2.3 Authentication of Herbal Material

All 17 selected herbs were purchased from a local supplier in Hong Kong and authenticated by a morphological expert, Dr. Cao Hui (National Engineering Research Center for Modernization of TCM, Zhuhai, Guangdong, China). Herbarium voucher specimens were deposited at the museum of the Institute of Chinese Medicine, the Chinese University of Hong Kong, with the voucher specimen numbers shown in Table 2.1.

Chinese Herbs	Voucher Specimen Numbers
Cortex Moutan (牡丹皮)	2003-2456
Rhizoma Smilacis Chinensis (菝葜)	2003-2463
Rhizoma Alismatis (澤瀉)	2003-2455
Radix Paeoniae Alba (白芍)	2005-2632
Semen Platycladi (柏子仁)	2005-2629
Radix Codonopsis (黨參)	2005-2628
Herba Lophatheri (淡竹葉)	2005-2634
Radix Glycyrrhizae (甘草)	2005-2623
Radix Puerariae (葛根)	2005-2630
Ramulus Cinnamorni (桂枝)	2005-2631
Cortex Phellodendri (黃柏)	2005-2624
Rhizoma Copitidis (黃連)	2005-2587
Radix Ophiopogonis (麥冬)	2005-2625
Radix Trichosanthis (天花粉)	2005-2626
Radix Pseudostellariae (太子參)	2005-2627
Rhizoma Anemarrhenae (知母)	2005-2633
Semen Ziziphi Spinosae (棗仁)	2005-2635

Table 2.1 Voucher specimen numbers of seventeen Chinese herbs

2.4 Extraction Method

2.4.1 Material and Methods

Each herb was sliced into small pieces, and the weight was recorded before extraction. 200ml distilled water was added per 50mg herb, then refluxed for two hours. Extracted solution was collected and the herb was refluxed for two more hours with fresh 200ml distilled water, and a second extracted solution was collected. The two extracted solutions were combined together, centrifuged at 13000×*g* for 15 minutes, the supernatant was then collected and frozen at -80°C. The frozen solution was completely dried by freeze-drier and stored in desiccators. The weight of dried extract was recorded and the extraction yield was calculated.

2.4.2 Results

The percentage yield of each herb was shown in Table 2.2. The percentage yield of the herbs varied greatly from 13.87 % to 44.96%.

2.4 Discussion

Cortex Moutan, Rhizoma Smilacis Chinensis, Rhizoma Alismatis, Radix Glycyrrhizae, Radix Puerariae, Ramulus Cinnamomi, Rhizoma Coptidis, Radix Ophiopogonis, Radix Trichosanthis, Radix Pseudostellariae and Rhizoma

Anemarrhenae are published to have anti-diabetic effects (Li *et al.*, 2004b). As the active component(s) and the mode of actions of anti-diabetic effect of some herbs are unclear, further investigations including *in vitro* and *in vivo* studies are needed.

The raw herbs mentioned above were authenticated before any pharmacological studies were carried out. All of them have been proved to be genuine by an expert on plant morphology.

In the next 3 chapters, *in vitro* assays, *in vivo* assays and purification of active component(s) from the most potent anti-diabetic herbs will be discussed.

Chinese Herbs	Percentage Yield (%)
Cortex Moutan (牡丹皮)	21.00
Rhizoma Smilacis Chinensis (菝葜)	25.53
Rhizoma Alismatis (澤瀉)	23.89
Radix Paeoniae Alba (白芍)	19.09
Semen Platycladi (柏子仁)	20.22
Radix Codonopsis (黨參)	32.39
Herba Lophatheri (淡竹葉)	30.15
Radix Glycyrrhizae (甘草)	33.74
Radix Puerariae (葛根)	13.87
Ramulus Cinnamorni (桂枝)	30.67
Cortex Phellodendri (黃柏)	32.50
Rhizoma Copitidis (黃連)	40.88
Radix Ophiopogonis (麥冬)	44.96
Radix Trichosanthis (天花粉)	30.32
Radix Pseudostellariae (太子參)	26.20
Rhizoma Anemarrhenae (知母)	37.52
Semen Ziziphi Spinosae (棗仁)	26.65

Table 2.2 The extraction yield of seventeen selected herbs.

Chapter 3 *In vitro* Studies on Selected Traditional Chinese Herbs

3.1. Introduction

As mentioned in chapter 1, the pharmacological actions on anti-diabetes mainly focus on four actions:

a) Increasing insulin secretion;

b) Enhancing glucose utility in peripheral tissue and adipocyte;

c) Suppressing hepatic gluconeogenesis; and

d) Inhibiting carbohydrates uptake from intestine.

The first mechanism mainly targeted on the insulin deficiency problem in type 2 DM due to β-cell dysfunction; the second and third mechanisms were focused on insulin resistance of target tissues and so to improve glucose homeostasis; and finally the forth mechanism targeted on impaired glucose tolerance in type 2 DM by preventing postprandial surge of blood glucose level, and together with insulin resistance and impaired β-cell function (Li *et al.*, 2005). Most of the western drugs have been developed to exert their anti-diabetic effects based on these four mechanisms (Anjana Patel, 2003). My project will focus on the anti-diabetic effects of traditional Chinese herbs under these four mechanisms.

Four *in vitro* assays were chosen to demonstrate the anti-diabetic effects of each herb:

i) Human skin fibroblast Hs68 and mouse adipocyte 3T3-L1 – to investigate the enhancing effect of herbal extract on glucose uptake into peripheral tissues.

ii) Rat hepatoma cell H4IIE – to investigate the inhibition of herbal extract on hepatic gluconeogenesis.

iii) Rabbit brush border membrane vesicle (BBMV) – to investigate the suppressing effect of herbal extract on glucose absorption into intestine.

The relationship of these four *in vitro* models in anti-diabetic studies of the herbs will be discussed below in this chapter.

3.2 Hepatic Gluconeogenesis Studies

3.2.1 Introduction

The liver plays a central role in controlling glucose homeostasis by gluconeogenesis (glucose production) and glycolysis (breakdown of glucose). Gluconeogenesis is the synthesis of glucose from non-carbohydrate precursors (pyruvate, lactate, glycerol and amino acids). It is very important to maintain brain

glucose level. In a longer period starvation, glucose must be formed from non-carbohydrate sources for survival.

Three essential enzymes play important role in gluconeogenesis, namely glucose 6-phosphatase (G-6-Pase), fructose 1,6-bisphosphatase (F-1,6-BPase) and phosphoenolpyruvate carboxylkinase (PEPCK). The process of gluconeogenesis is shown in Fig. 3.1 (Stryer L, 1995).

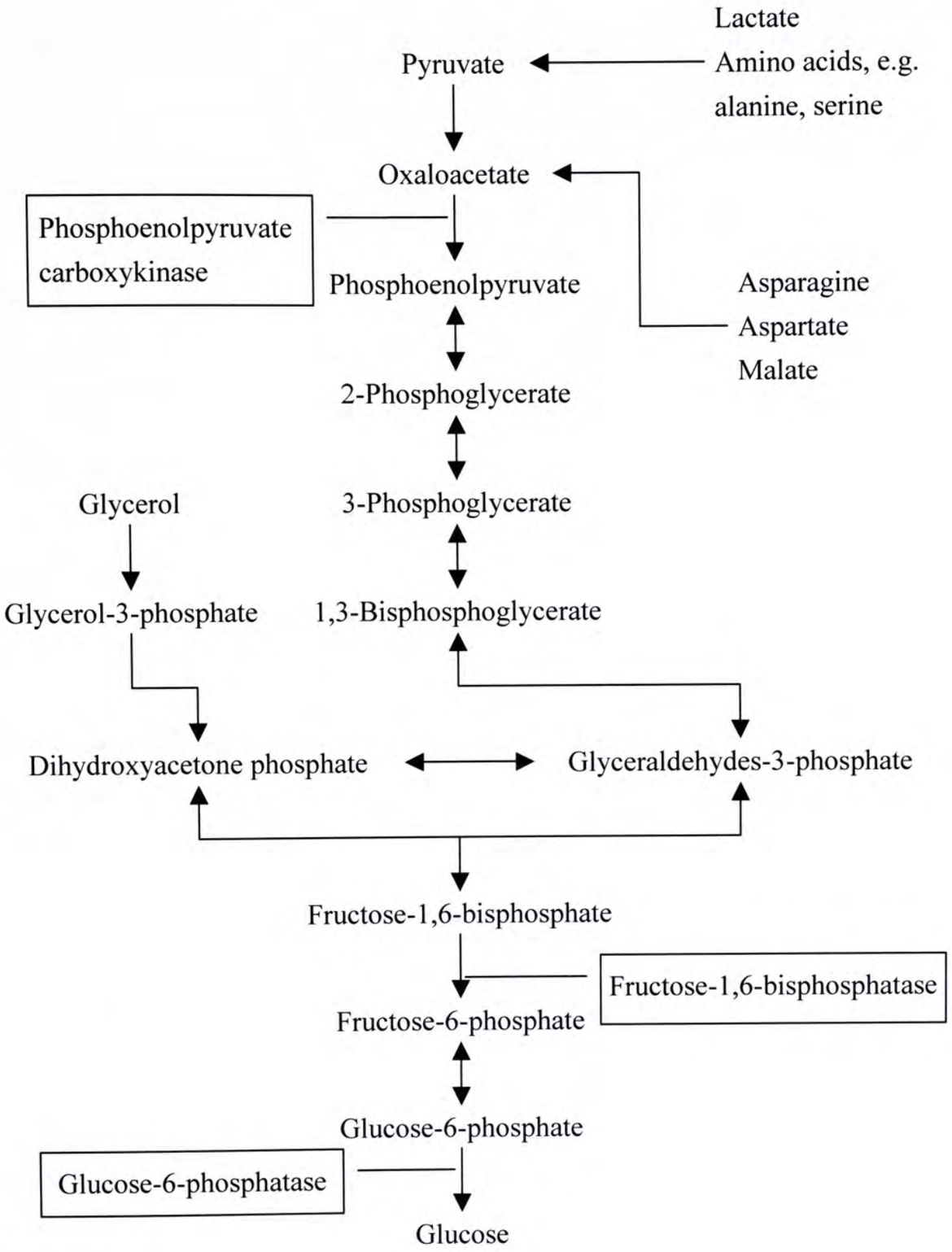


Fig. 3.1 The Gluconeogenesis Pathway. There are three major enzymes in gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase and glucose-6-phosphatase (Stryer L, 1995).

The blood glucose level at fasting stage is mainly maintained by glucose produced by liver, over 80% of blood glucose was released by glycogenolysis and gluconeogenesis (Stumvoll *et al.*, 1997), but in postprandial state, hepatic glucose output is suppressed by nearly 80% (Selz *et al.*, 2003). In type 2 diabetic patients, insulin resistance would lead to an increase of hepatic glucose output and blood glucose level as insulin could not exert its effect on controlling gluconeogenic enzymes (Barthel and Schmoll, 2003). Besides, during fasting state, basal hepatic glucose production is increased by 30% (Beck-Nielsen *et al.*, 2002; Staehr *et al.*, 2002). In postprandial state suppression of hepatic glucose output is lowered by 50% as excessive glucose is produced by gluconeogenesis in those patients (DeFronzo, 1999).

According to the above evidence, the study of inhibitory effect of drugs on hepatic gluconeogenesis can reveal the mode of action in diabetes treatment. Metformin is the most commonly used drug in treating abnormal hepatic glucose production in type 2 diabetic patients (Perriello *et al.*, 1994; Stumvoll *et al.*, 1995). H4IIE was used in this study, it is a cell line developed from rat hepatoma (Reauber, 1961; Pitot *et al.*, 1964). Moreover, the glucose produced by H4IIE could not be metabolized nor stored as glycogen, hence it is commonly used in gluconeogenesis study (Riu *et al.*, 1996). H4IIE retains the ability to convert lactate and pyruvate to

glucose, by expressing gluconeogenic enzymes including glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK). It also responses to various hormones such as insulin (which suppresses gluconeogenesis), adenosine 3',5'-cyclic monophosphate (cAMP) and dexamethasone (which induce gluconeogenesis) (Wang *et al.*, 2000; Hornbuckle *et al.*, 2001; Sasaki *et al.*, 1984).

G-6-Pase catalyzes the hydrolysis of glucose-6-phosphate (G-6-P) to glucose, which is the terminal step of hepatic gluconeogenesis and glycogen breakdown. G-6-Pase is induced in staved or diabetic animals (Nordlie *et al.*, 1999). A suitable anti-diabetic agent should have actions similar to insulin, and bypass the defects in insulin action characterized by insulin resistance (Schmoll *et al.*, 2000).

In this study, glucose production assay was applied to study the inhibitory effect of TCM. Dexamethasone and 8-(4-chlorophenylthio)-cAMP (pCPT-cAMP, a non-metabolized derivative of cAMP) were added to stimulate the gluconeogenic activity in H4IIE. Insulin was added in the assay as positive control as it could counteract the glucose production effect. As effective anti-diabetic agents should have a similar action compare to insulin, herbal water extracts were added in different concentrations in order to mimic the effect of insulin, and their inhibitory effects in gluconeogenesis were studied. Glucose produced was measured by the glucose oxidase method (Wang *et al.*, 2000; Waltner-Law *et al.*, 2002).

3.2.2 Material and Methods

3.2.2.1 Cell Culture of H4IIE

Rat hepatoma cell line H4IIE (American Type Culture Collection, ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) (supplemented with 4500mg/L D-glucose, 4mM L-glutamine, 110mg/L sodium pyruvate and 4mg/L pyridoxine HCl, pH.7.4), with 10% v/v fetal bovine serum (FBS), 5 ml streptomycin (100μg/ml) and penicillin (100 unit/ml) mixture solution. The cells were allowed to grow in a humidified incubator at 37°C with 5% CO_2. The medium was changed two to three times a week and cells were subcultured when the density reached 70% to 80%. Subcultivation was made by first removal of all the medium inside the culture flask, and a rinse with 10ml phosphate buffered saline (PBS, containing 137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, pH7.4). The solution was removed and 1 to 2 ml of trypsin-EDTA solution was added. The flask was allowed to sit at 37°C incubator for five minutes until the cells detach. Five mililitres of fresh DMEM was added to stop the reaction, and the cells were aspirated and dispensed back into culture flask in a subcultivation ratio 1:2 to 1:4. The frozen stocks of cells were stored with 95% culture medium and 5% DMSO under -80 °C. The cells were maintained between 4^{th} and 20^{th} passages for experiments.

3.2.2.2 Glucose Production Assay

Glucose production assay were carried out according to the method published by Waltner-Law *et al.*, 2002 and Wang *et al.*, 2000. 1.2×10^7 cells were seeded in 100mm dishes. After two days growth period, seeded cells were divided into three groups (with n=3 for each set of experiment): positive control, negative control and herbal water extracts treatment group. Then, the medium inside the dishes was removed and replaced by 3ml DMEM containing 500nM dexamethasone (DEX) and 0.1mM pCPT-cAMP. For negative control group only prepared medium was added; for positive control group, 10nM insulin was added together with prepared medium; and finally for herbal water extracts treatment group, different herbal extract concentrations (5, 1, 0.2mg/ml) were added together with prepared medium. The herbal extracts first need to dissolve into prepared medium followed sterilization and filtration of non-dissolved debris by 0.22μm filter (Millipore, Billerica, Mass, USA). After incubating for five hours, the medium was removed and the dishes were washed with PBS for three times. Two milliliters of glucose production buffer (glucose-free DMEM without phenol red, 5mM sodium pyruvate, 50mM sodium lactate) was then added in each dish. After three more hours incubation, the concentration of glucose produced by the cells was measured with use of an enzymatic-spectrophotometric glucose oxidase/peroxidase method, in which 1ml

buffer was aspirated and added into 0.3ml glucose assay solution (BioSystems, Barcelona, Spain), and incubated at 37°C for ten minutes. Absorbance at OD 500nm was recorded. After that, dishes were washed with PBS, and the cells were lysed with 5ml 0.5M NaOH for ten minutes. The lysate was neutralized with 5ml 0.5M HCl. The protein concentration of each dish was determined by bicinchoninic acid protein (BCA) assay (to be discussed in section 3.2.2.3). The amount of glucose produced by cells was normalized by their protein concentrations. Results were expressed as the percentage of glucose production of the negative control.

For statistical analysis, Mann-Whitney tests were used for comparisons of glucose production in H4IIE cells between control group and each herbal extract treatment group. Statistical tests were two-sided, with a significant level of 0.05.

3.2.2.3 Bicinchoninic Acid (BCA) Protein Assay

Working assay reagent was freshly prepared by mixing BCA Reagent A (Bio-Rad, Hercules, CA, USA) and Reagent B (4% $CuSO_4 \cdot 5H_2O$) in a ratio of 49:1. Then, 30µl of each sample and also bovine serum albumin (BSA) standard (0 – 10 mg/ml) were added into 96-well plate wells, after with 200µl working assay reagent. After incubation at 37°C for 30 minutes, OD 540nm was measured with Bio-Rad (Hercules, CA, USA) 3550 microplate reader. The sample protein concentration was determined by

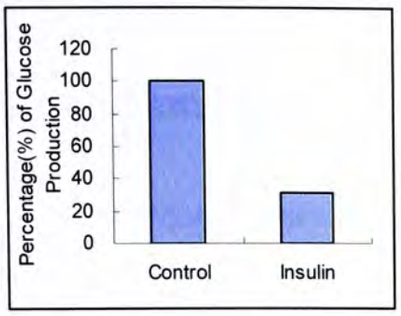
the calibration curve of an albumin standard.

3.2.3 Results

Results of glucose production assay are shown in Fig. 3.2a and Fig. 3.2b. In Fig. 3.2a, it showed that insulin, which acted as positive control, inhibited glucose production of H4IIE by 69% compared with that of control, and thus validated the model.

Among all tested herbs, Cortex Phellodendri (5mg/ml), Rhizoma Copitidis (1mg/ml and 5mg/ml) and Radix Pseudostellariae (5mg/ml) showed the most promising inhibitory effect on glucose production, which were 81.6%, 64.1%, and 88.8% inhibition respectively. Some of the other herbs also had significant inhibitory effects on gluconeogenesis, while some of them were found to have a stimulatory effect on gluconeogenesis.

(a)



(b)

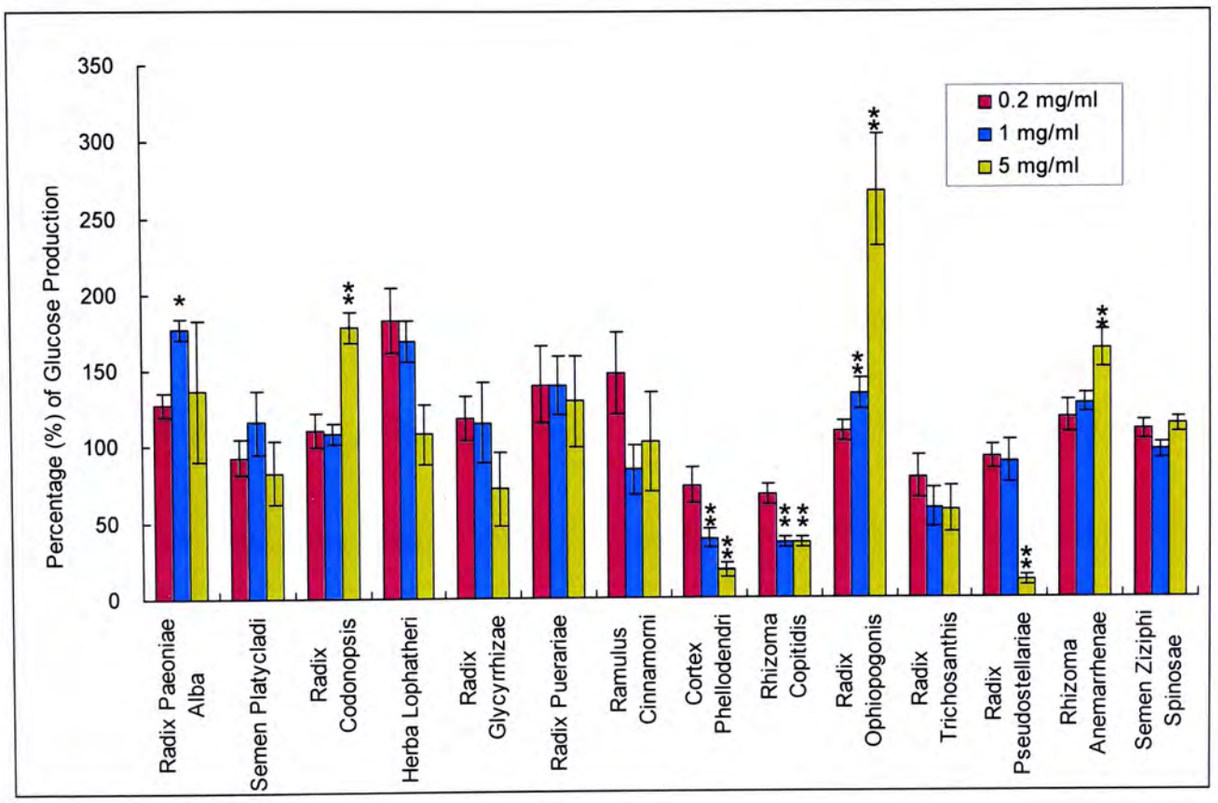


Fig. 3.2 Effects of herbal water extract treatments on gluconeogenesis in H4IIE hepatoma cells. The effect of insulin on gluconeogenesis was shown in (a); and that of the herbal water extracts was shown in (b). Cells were incubated with different herbal water extracts, or with 10nM of insulin in medium with DEX/pCPT-cAMP, then glucose production buffer containing gluconeogenic precursors was added, followed by determination of glucose released into the glucose production buffer by glucose oxidase assay. The amount of glucose produced was normalized by cell protein. The glucose production values of the herbal extract treated group were expressed as the percentage of the untreated control group (which is 100%). Data are expressed as mean ±SD (n =9). Significantly different from water control group: *p<0.05, **p<0.01, ***p<0.001.

3.3 Intestinal Glucose Absorption Studies

3.3.1 Introduction

Carbohydrates are our major energy source. Small intestine is the major organ in our body which absorbs carbohydrates from daily food intake (Sala-Rabanal *et al.*, 2004). There are many villi on the surface of intestine lumen to increase the surface area for nutrients absorption. Brush border membrane is found on the tips of the microvilli (on the surface of villi). The functional elements of the brush boarder membrane provide for the terminal digestion of carbohydrates and oligopeptides as well as their absorption of monosaccharides and amino acids (Schmitz *et al.*, 1973). Brush border membrane vesicle (BBMV) was first successfully purified from brush border membrane of human small intestine in 1973 (Schmitz *et al.*, 1973) and the purification method was further improved in 1978 by using Mg^{2+} precipitation method (Kessler *et al.*, 1978). The final product has no DNA, no other metabolic enzymes, but presented mainly Na^{+}/D-glucose cotransporter 1 (SGLT1) (Kessler *et al.*, 1978; Schmitz *et al.*, 1973). Over 90% of BBMV have the right orientation, exterior side allocates with SGLT1 and interior side (lumen) has no metabolic enzymes, hence solute being absorbed by transporters would not be metabolized and allowed for accumulation inside (Haase *et al.*, 1978). The glucose uptake of BBMV is well-characterized, SGLT1 is the transporter responsible for glucose absorption,

by transporting two Na^+ with one D-glucose molecule in the expense of ATP (Semenza *et al.*, 1984; Kessler *et al.*, 1978). As a result, BBMV was commonly used as *in vitro* research subject of glucose absorption.

For type 2 diabetic patients, the regulatory mechanisms of glucose homeostasis were defective due to insulin resistance and insulin deficiency: glucose disposal into peripheral tissues are defective, hepatic glycogen storage from glucose is lowered, also gluconeogenesis rate is increased at the postprandial stage (Gerich, 1997). Western drugs such as acarbose and α-glucosidase inhibitor are used to control glucose absorption by competitively inhibiting the ability of α-amylase and the α-glucosidase enzymes on the small intestinal brush border to break down oligosaccharides and disaccharides into monosaccharides (DeFronzo, 1999). In the present study, inhibitory effect of herbal hexane or dichloromethane extracts on glucose absorption through the small intestine was investigated. By inhibiting the glucose absorption, postprandial blood glucose level surge could be controlled.

3.3.2 Material and Methods

3.3.2.1 Preparation of BBMV

3.3.2.1.1 Chemicals

Here is the list of chemicals used in this experiment:

1. Purchased from Sigma (St. Louis, MO, USA):

   Potassium chloride (KCl), mannitol, 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$), sodium thiocyanate (NaSCN), sodium chloride (NaCl), phlorizin (phloretin 2'-glucoside, also known as phloridzin) and copper(II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$).

2. Purchased from Amersham (Little Chalfont, UK):

   D-[2-^{3}H]-glucose

3.3.2.1.2 Method

BBMV was prepared by the Mg^{2+} precipitation method (Kessler *et al.*, 1978; Schmitz *et al.*, 1973). A New Zealand white rabbit was scarificed and the small intestine was freshly dissected from the pyloric sphincter to ileocaecal junction. Firstly, the dissected intestine was immersed in 200ml ice-cold 154mM KCl. Secondly, all the fat tissues and mesentery were removed clearly and the content of

intestine was gently squeezed out. Thirdly, the intestine was cut longitudinally and washed thoroughly with 154mM KCl. The cut intestine was placed onto paper towers and blotted dry, and then it was wrapped with aluminum foil and snap frozen in liquid nitrogen. The frozen intestine was stored at -80°C.

To prepare BBMV, frozen small intestine was thawed in 200ml Buffer 1 (10mM mannitol, 2mM HEPES-Tris, pH 7.1) and was cut into small pieces, followed with mechanically vibrateion for one minute. The solution obtained was filtered under vacuum to remove connective tissues and muscles. Then the filtrate was made up to 300ml with Buffer 1. After that, 0.61g $MgCl_2$ was added to precipitate nuclei, mitochondria, most of basolateral membranes and other cell debris. The solution was centrifuged at 4°C, 3800×*g* for 15 minutes. The pellet was discarded, and the supernatant was collected and further centrifuged at 4°C, 43000×*g* for 30 minutes. The supernatant was discarded, and the pellet was resuspended in Buffer 2 (100mM mannitol, 0.1mM $MgSO_4$, 2mM HEPES-Tris, pH 7.4). The solution was centrifuged at 4°C, 43000×*g* for 40 minutes and the pellet was resuspended in 1ml Buffer 3 (300mM mannitol, 10mM HEPES-Tris, 0.1mM $MgSO_4$, pH 7.4). The volume of pellet was doubled using Buffer 3. The solution was passed through a 25 gauge needle five times to form vesicles. The solution containing BBMV was then divided into 0.26ml aliquots and snaps frozen in liquid nitrogen.

3.3.2.2 Preparation of Herbal Extracts

BBMV was used to study the effect of herbal extracts on intestinal glucose absorption. In this experiment, BBMV was incubated with radioactive glucose and herbal extract only. As the glucose concentration in the final BBMV mixture was only 0.067mM, so any glucose present in herbal extracts would affect the result by showing false positive. Hence, glucose inside herbs must be cleared by using an organic extraction method. However, many polar compounds were inevitably excluded by using this method.

Each herb was sliced into small pieces, and the weight was recorded before extraction. 200ml absolute ethanol (100% EtOH) was added per 50mg herb, then refluxed for two hours. The extracted solution was collected and the herb was refluxed for two more hours with fresh 200ml absolute ethanol, and a second extracted solution was collected. The two extracted solutions were combined together. After that, the solution was filtered through filter paper, and collected in a pre-weighed 250ml round bottom flask. The solvent inside the filtrate was evaporated using a rotary evaporator, and the dried content was weighed.

The ethanolic extract was re-dissolved in 100ml distilled water and partitioned with 100ml hexane (Lab-Scan, Bangkok, Thailand). The hexane fraction was collected, and the water fraction was partitioned again with 100ml of fresh hexane.

Two hexane fractions were combined in a pre-weighed round bottom flask. The remaining water fraction was partitioned with 100ml dichloromethane (CH_2Cl_2) (Lab-Scan, Bangkok, Thailand). Dichloromethane fraction was collected in a pre-weighed round bottom flask, and the water fraction was partitioned once again with 100ml of fresh dichloromethane. Again, two dichloromethane fractions were combined in the same flask. Anhydrous sodium sulphate (MERCK, Darmstadt, Germany) was added to absorb any remaining moisture in both hexane and dichloromethane extracts. The two solutions were filtered and the two extracts were dried using a rotary evaporator. The weights of two fractions were recorded. The dried fractions were stored in a desiccator.

3.3.2.3 BBMV Glucose Uptake Assay

A rapid filtration technique was used in this assay (Hopfer *et al.*, 1973). A time profile of each batch of prepared BBMV was made before it was used in the experiment as a quality control measure; also by this method the optimal time point for BBMV glucose uptake in the assay could be found. The batch of BBMV was proven to be functional if glucose uptake increased sharply with time peaking at around 10-30 seconds, and decreased gradually until 120 seconds.

Firstly, BBMV was thawed and resuspended in 0.44ml of Buffer 3. Then, 20µl

of diluted BBMV was mixed with 40μl of radiolabelled glucose solution (10μCi/ml D-[2-^{3}H]-glucose, 0.1mM D-glucose, 100mM NaSCN, 100mM mannitol, 10mM HEPES-Tris, pH 7.4). The reaction was stopped after specific incubation time (0, 10, 20, 30, 40, 60 and 120 seconds) by adding 1ml ice-cold stop-wash buffer (200mM NaCl, 10mM HEPES-Tris, 250μM phlorizin, pH 7.4), containing phlorizin as the SGLT1 inhibitor (competitive inhibitor). The reaction mixture was rapidly filtered through a pre-wetted 0.45μm cellulose nitrate filter (Millipore, Billerica, Mass, USA) under vacuum. The filter was washed five times with 1ml stop-wash buffer to wash away the non-specifically bound radiolabelled glucose, and BBMV which absorbed radioactive D-glucose was retained on the filter. The filter was then placed inside a scintillation vial with 4ml OptiPhase HiSafe 2 scintillation fluid (PerkinElmer life sciences, Inc., Boston, MA, USA). Radioactivity was counted by scintillation counter for one minute. The value of 0 second, representing the non-specific binding of glucose, was subtracted from all samples for the correction.

The protein concentration of BBMV suspension was determined. One aliquot of frozen BBMV (0.26ml) was resuspended in 0.44ml of water. Various dilutions (10-fold, 50-fold and 100-fold) were prepared, and the protein concentrations were determined by BCA assay (to be mentioned in section 3.3.2.4). Glucose uptake of BBMV was normalized with a standard curve using different concentrations of

BBMV protein, and expressed as pmol glucose uptake per mg protein.

To screen the inhibitory effect of glucose absorption of each herbal extracts, the experiment was performed in the same way, but at fixed time points. Both hexane and dichloromethane herbal extracts were dissolved in 100% EtOH in a concentration of 20mg/ml. Then, 20µl of diluted BBMV was mixed with 10µl of 0.4mM radioactive glucose solution (40µCi/ml D-[2-^{3}H]-glucose, 0.4mM glucose, 400mM NaSCN, 400mM mannitol, 40mM HEPES-Tris, pH 7.4) and 30µl of herbal extract (diluted to 1mg/ml by water, and the final EtOH concentration was equal to 4%). A control group was also made by using 4% v/v absolute EtOH in lieu of herbal extract, so as to compare with the effect of the herbal extracts. Glucose uptake was stopped after 20 seconds incubation (the optimum time of BBMV glucose uptake was investigated in the profile, i.e. 20 seconds) by adding 1ml of ice-cold stop-wash buffer. The glucose uptake by BBMV was measured as described previously in this section.

For statistical analysis, Mann-Whitney tests were used for comparisons of glucose absorption of BBMV between the control group and each herbal extract treatment group. Statistical tests were two-sided, with a significant level of 0.05.

3.3.2.4 Bicinchoninic Acid (BCA) Protein Assay

BCA protein assay was prepared as described in section 3.2.2.3. The Sample protein concentration was determined by the calibration curve of the albumin standard. It is used to normalize the radioactive count of glucose absorption.

3.3.3 Results

A typical glucose uptake profile of BBMV is shown in Fig. 3.3. The bell shape with a peak at 20 second showed it had the highest glucose penetration across the SGLT1 at that time and the glucose absorption decreased to one-tenth of the peak value at 3600 seconds, which proved the batch of BBMV was capable for use (Kessler *et al.*, 1978; Semenza *et al.*, 1984).

Results of BBMV glucose uptake assay were shown in Fig. 3.4. From the results of herbal dichloromethane extract treatment (1mg/ml), Radix Glycyrrhizae, Radix Ophiopogonis and Radix Trichosanthis showed the most potent and significant inhibitory effect on intestinal glucose absorption, with 82.8%, 93.1% and 81.2% inhibition respectively. And for hexane extract herbal treatments, Radix Glycyrrhizae, Radix Ophiopogonis and Radix Trichosanthis exerted the most potent significant inhibitory effect on intestinal glucose absorption, with 78.4%, 98.1% and 95.43% respectively. All herbal extracts showed sufficient inhibitory effect in suppressing

BBMV glucose absorption except 1mg/ml of dichloromethane extract of Semen Ziziphi Spinosae. None of the herbal extracts exerted stimulatory effects on absorption.

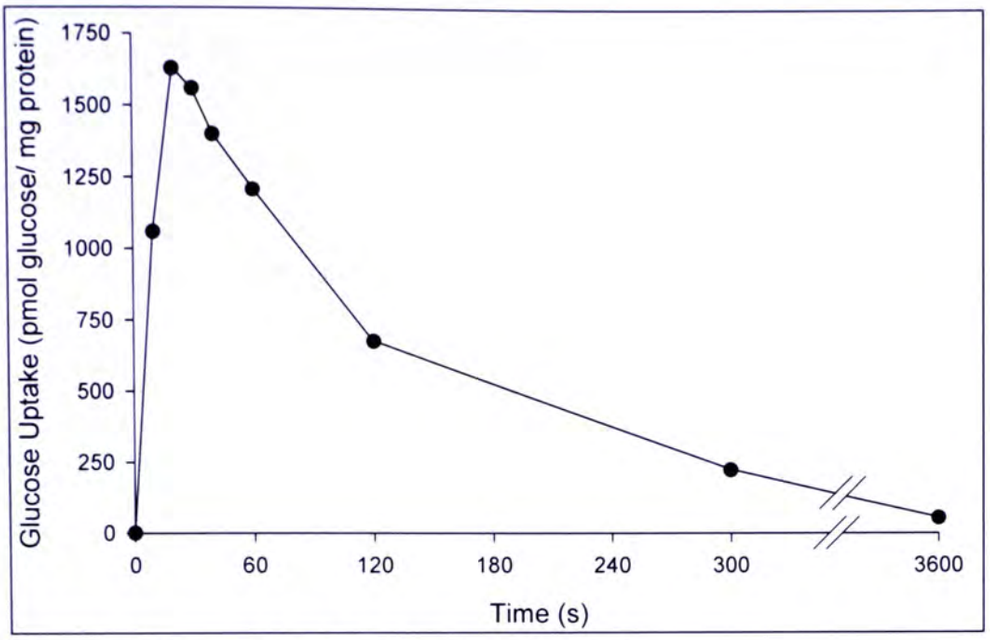


Fig. 3.3 Glucose Uptake Profile of BBMV

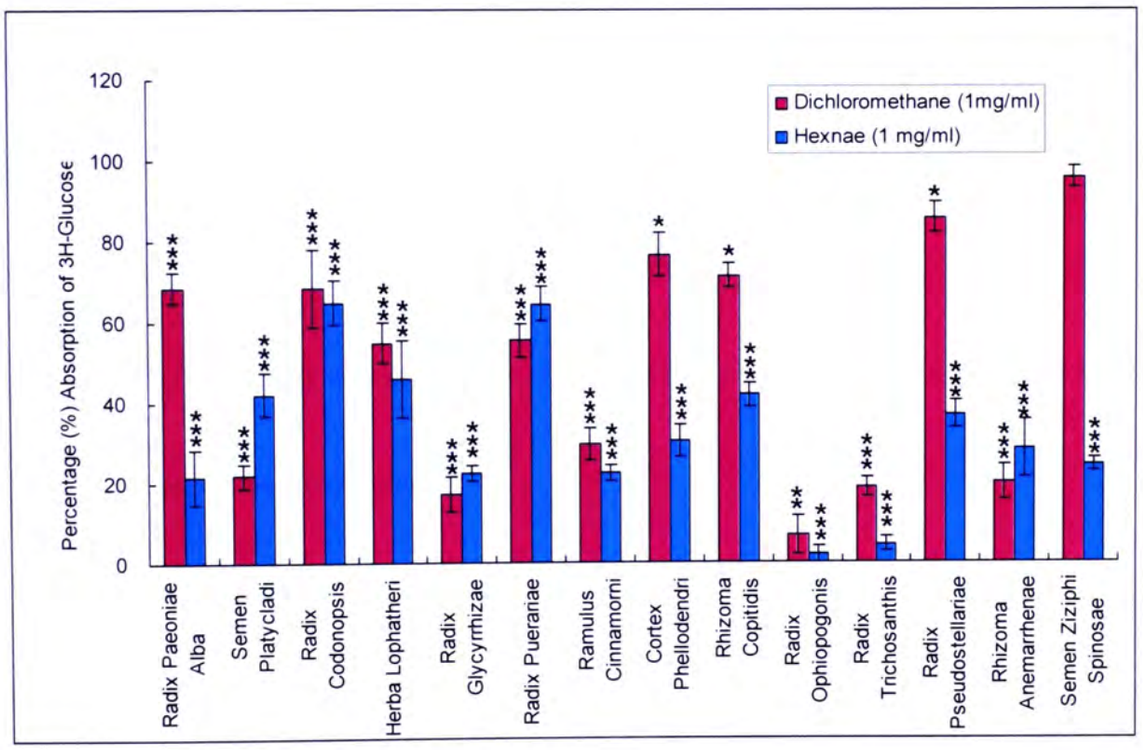


Fig. 3.4 Effects of herbal organic extract treatments on intestinal glucose absorption of BBMV. BBMV were mixed with ^{3}H-glucose and herbal dichloromethane or hexane extracts (1mg/ml) at room temperature and the glucose uptake reaction was stopped at 20 seconds by the addition of stop-wash buffer. The amount of radioactive glucose influx was normalized by the vesicle protein concentration. The glucose absorption enhanced by herbal extracts was expressed in percentage absorption compared with control (100%). Data are expressed as mean ±SD (n =9). Significantly different from 2% v/v EtOH control group: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.4 Fibroblast Glucose Uptake Studies

3.4.1 Introduction

Enhancing glucose uptake into peripheral tissue is one of the blood glucose homeostasis strategies. Cellular glucose uptake is facilitated by a family of glucose transporters (GLUT) (Bell *et al.*, 1990; Macheda *et al.*, 2005). Basal glucose uptake is mainly sustained by GLUT1, which is non-insulin dependent. GLUT1 is mostly present in fibroblasts. The basal glucose uptake in these peripheral tissues is lower than normal (Kahn, 2000). Hence, improving glucose uptake in fibroblasts is beneficial to maintaining glucose homeostasis in type 2 diabetic patients. In the present study, human fibroblast cell line Hs68 was employed for investigation of the effect of herbal water extract on their enhancing effect in insulin independent basal glucose uptake into fibroblast.

Hs68 is a human foreskin fibroblast cell line developed from an apparently normal newborn male (Corcoran and Stetler-Stevenson, 1995). It provides a model for non-insulin dependent glucose uptake study on GLUT1 (Gherzi *et al.*, 1992). Fibroblasts provide a convenient model for glucose transport study as it composes 15% of an individual's total body weight, hence a reduction of the glucose uptake into skin cells could lead to increased glucose levels in the blood (Gherzi *et al.*, 1992).

2-deoxy-D-glucose (2-DOG), which is a glucose derivative, could not be metabolized by the cell, is used in glucose uptake study instead of glucose. It is because 2-deoxy-D-glucose undergoes phosphorylation inside the cells and cannot be further metabolized into the glycolytic intermediates (Navon *et al.*, 1989).

3.4.2 Material and Methods

1. Purchased from Sigma (St. Louis, MO, USA): 2-deoxy-D-glucose (2-DOG)
2. Purchased from Gibco BRL Life Technologies, Inc. (Carlsbad, CA, USA): Glucose-free DMEM
3. Purchased from Amersham (Little Chalfont, UK): 2-Deoxy-D-[1-^{3}H]-glucose (2-DOG)

3.4.2.1 Cell Culture of Hs68

The cell culture procedures for human skin fibroblasts Hs68 (ATCC, Manassas, VA, USA) were the same as that of H4IIE cultures (refer to section 3.2.2.1). In this study, Hs68 was maintained between 4th and 20th passages.

3.4.2.2 2-Deoxy-D-glucose Uptake Assay

The assay was performed as described by Jarvill-Taylor *et al* (2001). Hs68 fibroblasts were allowed to grow to a confluent state in 24-well plates. Hs68 cells were then glucose-starved by glucose-free DMEM for 1 hour. Cells were divided into a negative control group (glucose-free DMEM), and herbal treatment groups (10, 1, 0.1 and 0.01mg/ml herbal water extracts dissolved in glucose-free DMEM, filtered with 0.22μm filter (Millipore, Billerica, Mass, USA)). Cells were incubated at 37°C for 30 minutes. The cells were then washed with PBS for three times to remove the herbal extracts. Three hundred microliters radiolabeled 2-DOG mixture (2μCi/ml 2-deoxy-D-[1-^{3}H]-glucose, 0.1mM 2-DOG in glucose-free DMEM) was added and incubated for 15 min at 37°C. Reactions were then stopped by adding 1ml/well of ice-cold 10mM 2-DOG solution, and each well was washed twice more by the same ice-cold 10mM 2-DOG solution to remove any unbound radioactive glucose. A non-specific binding control was performed in each experiment set, in which no treatment was applied and radioactive 2-DOG mixture was added after adding ice-cold 2-DOG into the wells. It was used to determine the non-specific binding of the radioactive 2-DOG on the cell membrane surface. Then, 100μl of 0.5M NaOH was added to lyse the cells, and equal amount of 0.5M HCl was then added to neutralize the cell lysate. Afterwards, 120μl of the cell lysate was transferred into scintillation vials and 4ml of

OptiPhase HiSafe 2 scintillation fluid (PerkinElmer, Boston, MA, USA) was added for radioactivity counting by a liquid scintillation counter. Protein content of samples was determined by BCA assay (section 3.2.2.3).

The radioactive count of glucose uptake was corrected by subtracting the mean of non-specific binding values, and then the amount of 2-DOG uptake was corrected by the protein amount. Results were expressed as the percentage of 2-DOG uptake compared with that of the negative control.

For statistical analysis, two-sided Mann-Whitney tests were performed for comparisons of 2-DOG uptake between control and treatments, with a significant level of 0.05.

3.4.2.3 Bicinchoninic Acid (BCA) Protein Assay

BCA protein assay was carried out as described in section 3.2.2.3. The sample protein concentration was determined by the calibration curve of albumin standard. It is used to normalize the radioactive count of glucose uptake.

3.4.3 Results

The results in Fig. 3.5 showed that most of the 10mg/ml herbal extract gave significant stimulation on radio-labelled 2-deoxyglucose uptake into Hs68 fibroblasts

through GLUT1. Radix Codonopsis (10mg/ml), Radix Paeoniae Alba (10mg/ml) and Semen Ziziphi Spinosae (0.01mg/ml) showed the most potent and significant enhancing effect on peripheral tissue glucose uptake, which increased the uptake by 114.1%, 133.9% and 220.4% respectively. For Radix Codonopsis it showed a dose dependent effect, while that of Semen Ziziphi Spinosae has an opposite effect (i.e. the higher of the dose, the smaller the effect). It might be due to toxicity of the herbal extract, hence the higher the dosage used, the more cells died and thus led to a decrease in 2-DOG uptake.

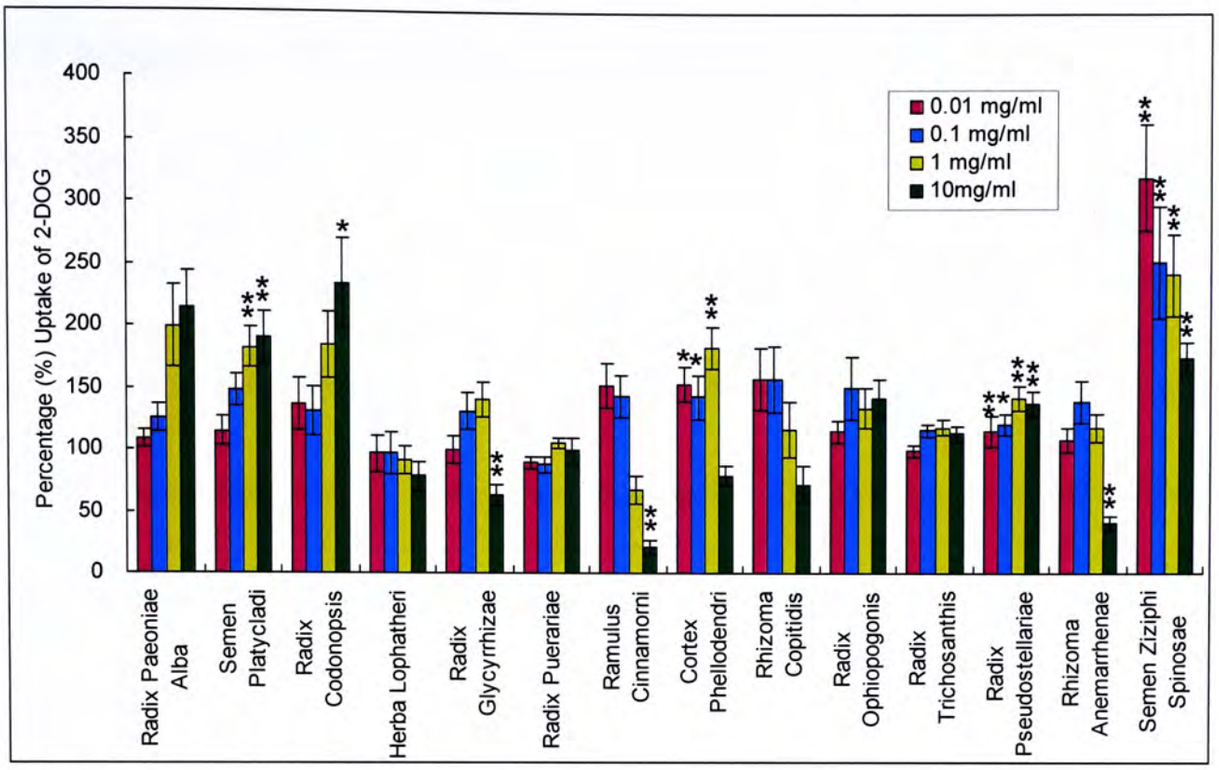


Fig. 3.5 Effects of herbal water extract treatments on glucose uptake of Hs68 human skin fibroblasts. Hs68 fibroblasts were incubated for 30min with different herbal water extracts, followed by the addition of 2-deoxy-D-[^{3}H]glucose for glucose uptake assays. The uptake reaction was stopped at 15min incubation by the addition of an ice-cold 2-DOG solution 10mM. The amount of radioactive glucose influx was normalized by cell protein concentrations. The glucose uptake values of the herbal water extract treated group were expressed as the percentage of the treated compared to that of untreated control group (100%). Data are expressed as mean ±SD (n =9). Asterisles were used to indicate significantly different results of treated groups compared with the water control group: *$p<0.05$, **$p<0.01$.

3.5 Adipocyte Glucose Uptake Studies

3.5.1 Introduction

Both skeletal muscle and adipose tissue are main tissues that response to blood glucose homeostasis (Li and Kandror, 2005; Rudich *et al.*, 2003). They express non-insulin dependent GLUT1 and insulin responsive GLUT4 on their cell surface. GLUT1 is responsible for the basal glucose transport while GLUT4 is responsible for the insulin-stimulated glucose transport in the adipocytes (Harrison *et al.*, 1992). GLUT4 is originally located inside the cytoplasm of adipocytes. In the basal state, most of the GLUT4 is sequestered in vesicular membranes inside the adipocyte. Upon insulin stimulation, GLUT4 translocation is triggered, and translocates on cell membrane, resulting in an increase in GLUT4 content on the cell surface which enhance glucose uptake (Hashiramoto and James, 2000; Walton *et al.*, 2004).

Once blood glucose level is high, insulin is released from the pancreatic islet, and glucose uptake through GLUT4 into skeletal muscle and adipocyte is stimulated. This could lower the blood glucose level and maintains it at a steady level (Proietto *et al.*, 1983). At an insulin resistance state of diabetic patients, glucose uptake into peripheral tissues is suppressed as insulin sensitivity of these cells is defective (Summers *et al.*, 1999). Moreover, the basal glucose uptake of the adipose tissue is lower than that of the normal state (Stolic *et al.*, 2002). Western oral medication

thiazolidinedione can target insulin-responsive tissues to enhance their glucose transport and improve their insulin sensitivity (Martens *et al.*, 2002). Hence, improving the glucose uptake in skeletal muscle and adipocytes is beneficial to maintain glucose homeostasis in type 2 diabetic patients.

Adipocytes were used as the model in this experiment for peripheral tissue regulating insulin-stimulated glucose homeostasis. The effects of the herbal water extracts on glucose uptake into these cells were studied. Mouse adipocyte 3T3-L1, which is a continuous substrain of 3T3 cell line, is an embryonic fibroblast cell line isolated from Swiss albino mouse (Green and Meuth, 1974). It can be induced into adipocytes from a pre-adipocyte state or from a rapidly dividing to a confluent and contact inhibited state by stimulation with 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX) and insulin (Green and Meuth, 1974). The morphology of full differentiated adipocytes is different from that of the fibroblasts and shows intracellular lipid accumulation (Wu *et al.*, 1998). 3T3-L1 adipocytes can perform insulin responsive glucose uptake by activation of insulin (Fong *et al.*, 1991). Glucose uptake assay was carried out similar to section 3.4.2.2 by means of 2-deoxy-D-gluose (2-DOG).

3.5.2 Material and Methods

1. Purchased from Sigma (St. Louis, MO, USA): Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, paraformaldehyde and 2-deoxy-D-glucose (2-DOG)
2. Purchased from Gibco BRL Life Technologies, Inc. (Carlsbad, CA, USA): Glucose-free DMEM
3. Purchased from Amersham (Little Chalfont, UK): 2-Deoxy-D-[1-^{3}H]-glucose

3.5.2.1 Cell Culture of 3T3-L1

The cell culture procedures for mouse fibroblast 3T3-L1 (ATCC, Manassas, VA, USA) were the as same as the culture method of H4IIE (refer to section 3.2.2.1). In this study, 3T3-L1 cells were maintained between 4th and 6th passages.

3.5.2.2 Differentiation of 3T3-L1

3T3-LI fibroblast cells were chemically induced into differentiated adipocytes before glucose uptake assay was carried out. Cells were seeded on the 24-well plates and maintained until after a confluent state for two days (Day 0). The medium was switched to IBMX-DEX-INS cocktail medium (0.5mM IBMX, 0.5μM dexamethasone and 5μg/ml insulin in complete DMEM, freshly prepared) at day 0.

On day 3, the IBMX-DEX-INS cocktail medium was removed and changed into the complete medium supplemented with 5μg/ml insulin. Two days later (day 5), the cells were maintained in normal complete medium (DMEM with 10% FBS, 100μg/ml streptomycin and 100unit/ml penicillin) and fed every other day. The cells were ready for use between day 9 and day 12.

Oil red O staining was applied to differentiated 3T3-L1 cells to detect the amount of lipid accumulation (i.e. to confirm the induction level of 3T3-L1). The cells were fixed with 2% paraformaldehyde (Sigma, St. Louis, MO, USA). Oil red O solution was prepared by mixing oil red O saturation isopropanol solution with distilled water in a ratio of three to two, and then allowed to stand in room temperature for 10 minutes, filtered immediately before used. This freshly prepared oil red O solution was added to the cells in 24-well plates, and allowed to stand in room temperature for 10 to 15 minutes. The solution was then washed away with PBS and the cells were observed under a light microscope. The lipid accumulation density in the differentiated cells should be around 90%.

3.5.2.3 2-Deoxy-D-glucose Uptake Assay

This assay was performed similar to the assay described in section 3.4.2.2. Differentiated 3T3-L1 cells were allowed to grow into a confluent state in 6-well

plates. 3T3-L1 cells were then serum-starved by glucose-free DMEM for 1 hour. Cells were divided into 2 sets (insulin-treated group and non-insulin-treated group), and each set contained a negative control group (glucose-free DMEM), and herbal treatment groups (10, 1, 0.1 and 0.01mg/ml herbal water extracts dissolved in glucose-free DMEM, filtered with 0.22μm filter (Millipore, Billerica, Mass, USA)). Different treatments were added to the cells and the cells were incubated at 37°C for 30 minutes. The cells were then washed with PBS for two times to remove the herbal extracts. Afterwards either set of the cells (insulin treated group) were incubated with 10nM insulin at 37°C for a further 30 minutes. Three hundred microliters radiolabelled 2-DOG mixture (2μCi/ml 2-deoxy-D-[1-^{3}H]-glucose, 0.1mM 2-DOG in glucose-free DMEM) was added and incubated for 15 minutes at 37°C. Reactions were then stopped by adding 1ml/well of ice-cold 10mM 2-DOG solution, and each well was washed twice more by the same ice-cold 10mM 2-DOG solution to remove any unbound radioactive glucose. Non-specific binding was performed in each experiment set, in which no treatment was applied and radioactive 2-DOG mixture was added after adding ice-cold 2-DOG into the wells. It was used to determine the non-specific binding of the radioactive 2-DOG on the cell membrane surface. Then, 200μl of 0.5M NaOH was added to lyse the cells, and equal amounts of 0.5M HCl was then added to neutralize the cell lysate. Afterwards, 300μl of the cell lysate was

transferred to scintillation vials and 4ml of OptiPhase HiSafe 2 scintillation fluid (PerkinElmer, Boston, MA, USA) was added for radioactivity counting by a liquid scintillation counter. The protein content of samples was determined by BCA assay (section 3.2.2.3).

The radioactive count of glucose uptake was corrected by subtracting the mean of non-specific binding values, and then the amount of 2-DOG uptake was corrected by protein amount. Results were expressed as the percentage of 2-DOG uptake compared with that of the negative control.

For statistical analysis, two-sided Mann-Whitney tests were performed for comparisons of 2-DOG uptake between control and treatments, also insulin-treated and non-insulin treated, with a significant level of 0.05.

3.5.2.4 Bicinchoninic Acid (BCA) Protein Assay

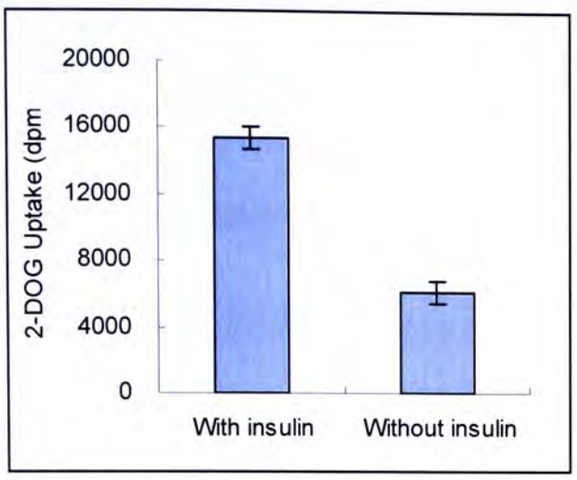
BCA protein assay was performed as described in section 3.2.2.3. The sample protein concentration was determined by the calibration curve of albumin standard. It was used to normalize the radioactive count of glucose uptake.

3.5.3 Results

In Fig. 3.6(a), it showed that the radiolabeled 2-DOG uptake was enhanced by insulin (act as positive control), therefore validated the model. The results were shown in Fig. 3.6(b). The insulin treatment group had inhibitory effect on radio-labelled 2-deoxyglucose uptake rather than stimulatory effect (except 10 mg/ml of Cortex Phellodendri) comparing that of non-insulin treatment group with the same concentration. Only Cortex Phellodendri (10mg/ml) exerted herb-insulin interaction in glucose uptake while other herbal extracts did not; and our results suggest that Cortex Phellodendri (10mg/ml) could improve the insulin sensitivity of the cells.

From the results, none of the herbal treatment exhibited a significant increase in glucose uptake into 3T3-L1 adipocytes. On the contrary, all tested concentration of Rhizoma Coptidis with insulin, Cortex Phellodendri with insulin (0.01mg/ml and 1mg/ml), Radix Ophiopogonis with insulin (1mg/ml and 10mg/ml) and Radix Ophiopogonis without insulin (1mg/ml and 10mg/ml) treatment groups significantly inhibited glucose uptake, which were unfavourable effects.

(a)



(b)

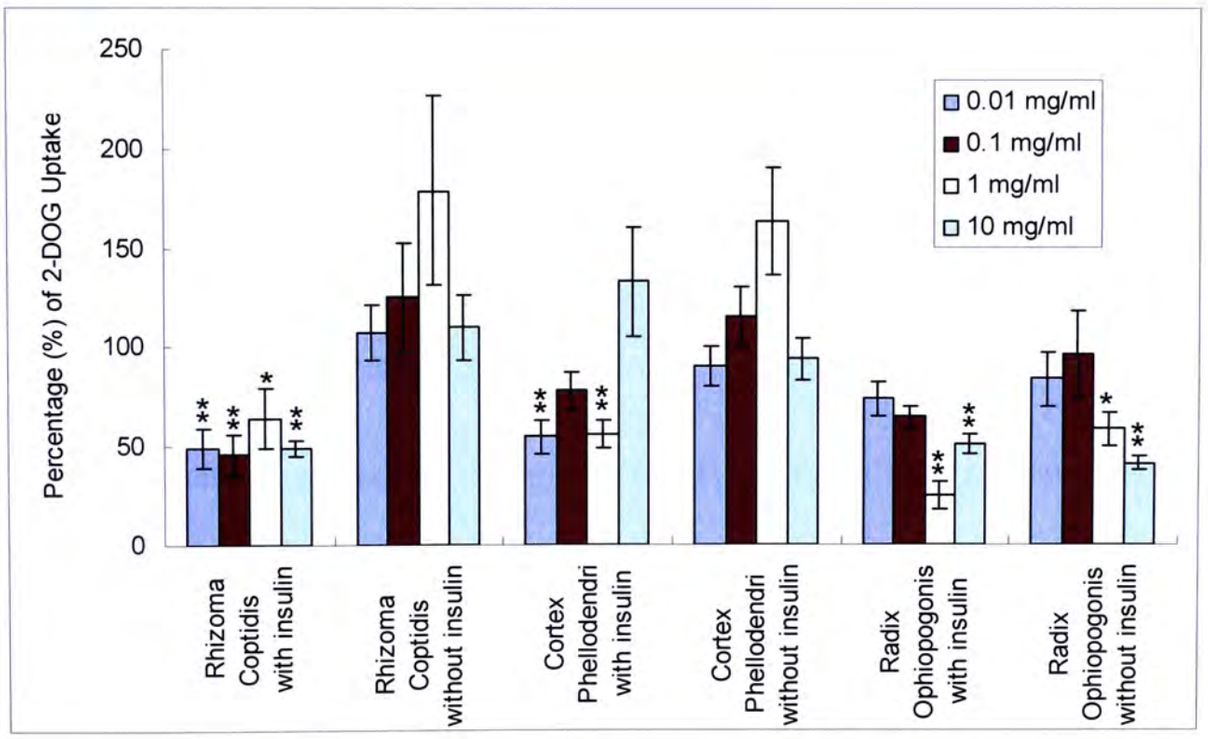


Fig.3.6 Effects of herbal water extract treatments on glucose uptake of 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 30mins with different herbal water extracts, and then incubated for another 30mins with 100mM insulin (for with insulin treatment groups). Then 2-deoxy-D-[^{3}H]glucose was added for glucose uptake assays. The uptake reaction was stopped at 15min incubation by the addition of an ice-cold 2-DOG solution 10mM. The amount of radioactive glucose influx was normalized by cell protein concentrations. The glucose uptake values of the herbal water extract treated group were expressed as the percentage of the treated compared to that of untreated control group (100%). Data are expressed as mean ±SD (n =9). Asteristo were used to indicate the significantly different results between each treatment group from water control group: *$p<0.05$, **$p<0.01$.

3.6 Glucose Transporter Type 4 (GLUT4) Expression Studies

3.6.1 Introduction

In type 2 diabetes, one of the main pathophysiologic defects is insulin resistance. In adipocytes, the decreased insulin sensitivity is due to a decreased expression of GLUT4 (Minokoshi *et al.*, 2003; Shepherd and Kahn, 1999). Both protein and mRNA levels of GLUT4 are decreased in adipocytes under an insulin resistance state, resulting in a change of glucose homeostasis (Minokoshi *et al.*, 2003).

3.6.2 Material and Methods

3.6.2.1 Cell Culture of 3T3-L1

The cell culture procedures for mouse fibroblasts 3T3-L1 (ATCC, Manassas, VA, USA) were the same as that of the culture method of H4IIE (refer to section 3.2.2.1). In this study, 3T3-L1 cells were maintained between 4^{th} and 6^{th} passages.

3.6.2.2 Differentiation of 3T3-L1

3T3-LI fibroblast cells were chemically induced into differentiated adipocytes before expression assay was carried out. The method was the same as that of 3T3-L1 adipocyte glucose uptake assay (refer to section 3.5.2.2).

3.6.2.3 GLUT4 Expression Assay

Differentiated 3T3-L1 cells were allowed to grow into a confluent state in 6-well plates. 3T3-L1 cells then were serum-starved by glucose-free DMEM for 1 hour. Cells were divided into 2 sets (insulin-treated group and non-insulin-treated group), and each set contained a negative control group (glucose-free DMEM), and herbal treatment groups (10, 1, 0.1 and 0.01mg/ml herbal water extracts dissolved in glucose-free DMEM, filtered with 0.22μm filter (Millipore, Billerica, Mass, USA)). Different treatments were added to the cells and the cells were incubated at 37°C for 30 minutes. The cells were then washed with PBS for two times to remove the treatments. Afterwards insulin treated group were incubated with 10nM insulin, and non-insulin treated group were incubated with glucose-free medium. Both treatment groups were incubated at 37°C for 30 minutes more.

3.6.2.4 Preparation of RNA

After treatment with different concentrations of herbal water extracts, RNA was purified from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Firstly, the medium from each well was removed, and 1ml TRIzol reagent was added into each well, and cells were incubated at room temperature for 5 minutes. The cell lysates were then pipetted into 1.5ml centrifuge tubes, 0.2ml of chloroform was added into

each tube and vigorously shaken for 15 minutes. Then, the tubes were centrifuged at 14000rpm for 20 minutes at 4°C. The upper colorless layer of the content (0.4ml) in the tubes was carefully removed to a clean centrifuge tube without disturbing the lower layer, and 0.4ml isopropanol was added to each tube and mixed well. After that, the tubes were centrifuged at 14000rpm for 20 minutes at 4°C. The supernatant was removed, and the white pellet was rinsed with cold 75% EtOH and air dried briefly. Forty microliters of DEPC-treated sterile distilled water (DEPC-H_2O) was added to each tube to dissolve the pellets. RNA was quantitated by UV absorbance at 260nm; in all cases the ratio of the UV absorbance at 260nm to that at 280 nm was greater than 1.5. Five micrograms of extracted RNA were used for first-strand cDNA synthesis.

3.6.2.5 RT-PCR

Five to ten micrograms of RNA was added to 2ul oligonucleotide (10pmol) and the final volume was adjusted to 37μl by DEPC-treated water. The reaction mixture was incubated at 65°C for 5 minutes and chilled on ice for 3 minutes. Reverse transcription was then performed at 42 °C for 1 hour with First-Strand Master Mix (5μl dNTP (10mM), 5μl 10X buffer, 1μl RNase inhibitor and 1μl PowerScript™ Reverse Transcriptase). The mixture was heated at 90°C for 5 minutes to inactivate

reverse transcriptase. 1ul RNase H was added to the first-strand reaction product and incubated at 37°C for 20 minutes to remove unused RNA.

3.6.2.6 PCR Analysis on GLUT4 Expression

For best results, the PCR cycling parameters were optimized. Choosing the optimal number of PCR cycles ensures that the double-stranded cDNA would remain in the exponential phase of amplification. Over-cycling of PCR reaction would lower the quality of cDNA template for subtraction while under-cycling would lower the yield of PCR product. The optimal number of PCR cycle was one cycle before reaching the plateau. 2 sets of PCR reaction were performed with first-strand cDNA product (5μl GLUT4 study group and 10μl for control group), 1μl of primer mix (10mM) (Forward primer sequence: 5' TGAACAATCTGAACGCACCGA 3'; Reverse primer sequence: 5' AGGAGAGCAGGGAGTACTGTGA 3') and 19.5μl of PCR Master Mix including 2μl of 10X PCR buffer, 0.5μl of magnesium chloride ($MgCl_2$), 1μl of dNTP (10mM) and 1μl of Taq polymerase, the final volume was adjusted to 20μl by distilled water. The reaction mixture was then subjected to 94°C denaturation for 5 minutes, followed by 30 cycles of amplification (94°C, 1 min30 sec; 65°C, 1min30 sec; 72°C, 1min30sec) and 72°C termination for 10 minutes. 10μl of each PCR product was then analyzed on a 1.0% TAE

agarose/ethidium bromide (EtBr) gel with a fixed voltage at 100V. After gel electrophoresis, the image was deteced under UV light and the band intensity was analyzed using a densitometer. The expression level of GLUT4 was normalized by the expression level of the housekeeping gene GAPDH.

For statistical analysis, student t-tests were performed for comparisons of between control and treatments, as well as those between control, insulin-treated and non-insulin treated, with a significant level of 0.05.

3.6.2.7 Real-time PCR

Real-time PCR was developed because it can investigate the quantitated differences in mRNA expression, and is particularly valuable when the amounts of RNA are low, since the fact that PCR involves an amplification step. PCR is the most sensitive method and can discriminate closely related mRNAs. But using conventional PCR method, it is difficult to obtain a truly quantitative result due to the limited sensitivity of ethidium bromide. In contrast to regular reverse transcriptase-PCR (RT-PCR) and analysis by agarose gels, real-time PCR gives a more quantitative result. An additional advantage of real-time PCR is the relative ease and convenience of use compared to some older methods.

The following paragraphs describe the experiment procedures. Total cellular RNA was extracted by TRIzol reagent and single-strand oligo-dT-primed cDNA was synthesized as described in section 3.6.2.4 and Real-time PCR was used to quantify GLUT4 mRNA expression.

The quantification was performed with the use of gene-specific primers, SYBR Green Universal PCR Mix and an ABI Real-time PCR 7700 machine (Applied Biosystems) starting with first-strand cDNAs. The quantity of GLUT4 gene in each sample was normalized by the corresponding GAPDH expression levels. The primer sequences for GAPDH amplification were 5'-ACCACAGTCCATGCCATCAC-3' (forward primer) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse primer). And the primer sequences for GLUT4 amplification were 5'-AGAGCTACAATGCAACGTGGCT-3' (forward primer) and 5'-AGGAGAGCAGGGAGTACTGTGA-3' (reverse primer). The reaction mixture was then subjected to PCR temperature profile of 50°C, 2min; 95°C, 10min; 95°C, 15sec; 60°C, 1min. Each sample was run in triplicate and the mean Ct value was used to calculate mRNA levels. The fold of change of mRNA level of each sample compared with control was calculated based on the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

For statistical analysis, student t-tests were performed for comparisons between control and treatments, as well as those between control, insulin-treated and non-insulin treated, with a significant level of 0.05.

3.6.3 Results

Fig. 3.7 shows the gel electrophoresis of PCR products of the GLUT4 gene and housekeeping GAPDH gene. Fig. 3.8 shows the band intensity of the DNA by gel electrophoresis. The band intensity was measured and expressed the percentage of GLUT4/GAPDH. The smaller the percentage, the higher is the GLUT4 gene expression inside 3T3-L1 adipocytes after different herbal treatments.

There was no significant increase in GLUT4 gene expression after 30 minutes herbal water extract treatment from RT-PCR.

The results were further confirmed by quantitative real-time RT-PCR method. Results in Fig. 3.9 showed that none of the herbal treatment group gave a significant increase in the level of GLUT4 gene expression relative to that of GAPDH gene, which illustrated that there was no increase in the level of GLUT4 gene after 30 minutes incubation with different herbal treatments.

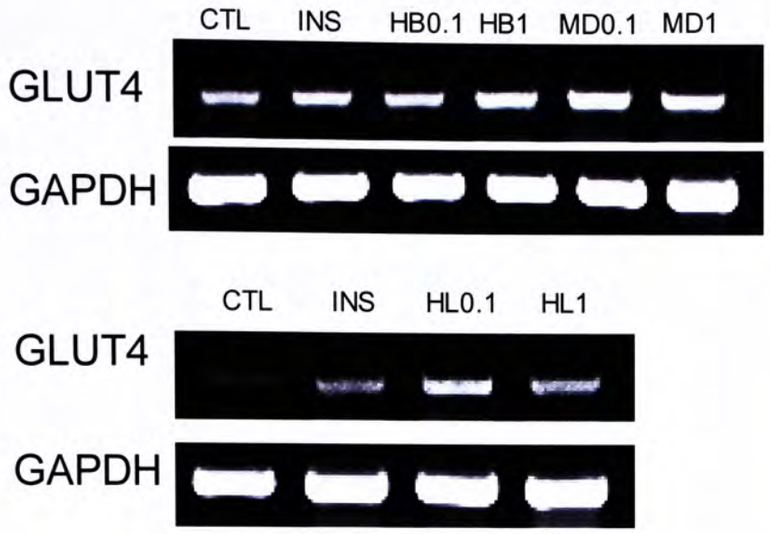


Fig. 3.7 The representative RT-PCR of GLUT4 expression. 3T3-L1 adipocytes were incubated for 30min with different herbal water extracts, then another 30min with 100mM insulin (for with insulin treatment groups).The total RNA was extracted from herbal water extract treated 3T3-L1 adipocytes and reverse transcribed, the product was amplified by PCR and then separated by agarose gel electrophoresis and detected with ethidium bromide staining. The gene expression of GLUT4 was compared with that of housekeeping gene GAPDH (n=3).

HB 0.1 = Cortex Phellodendri (0.1mg/ml)
HB 1 = Cortex Phellodendri (1mg/ml)
MD 0.1= Radix Ophiopogonis (0.1mg/ml)
MD1= Radix Ophiopogonis (1mg/ml)
HL 0.1= Rhizoma Coptidis (0.1mg/ml)
HL 1= Rhizoma Coptidis (1mg/ml)

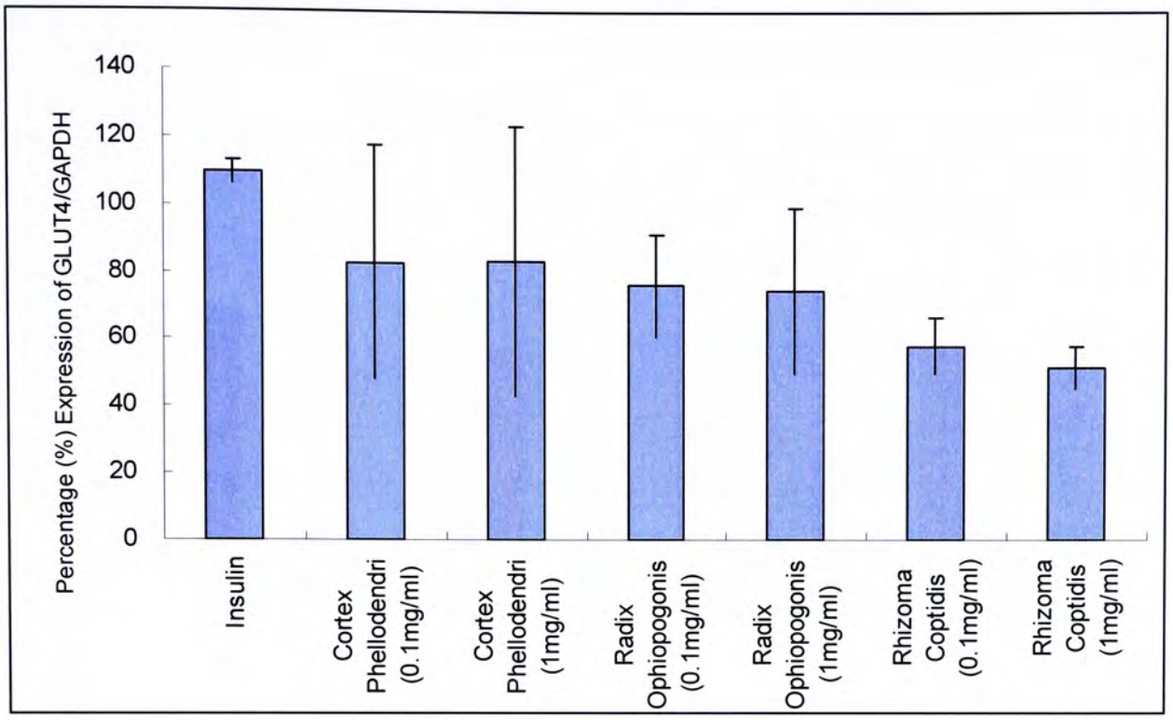


Fig. 3.8 GLUT4 gene expression in 3T3-L1 adipocytes. Percentage ratio of GLUT4 mRNA relative to GAPDH mRNA was calculated. The results were compared with control group without any treatment (100%). Data was expressed as mean ±SD (n=3). There is no significant increase in gene expression among all treatment groups.

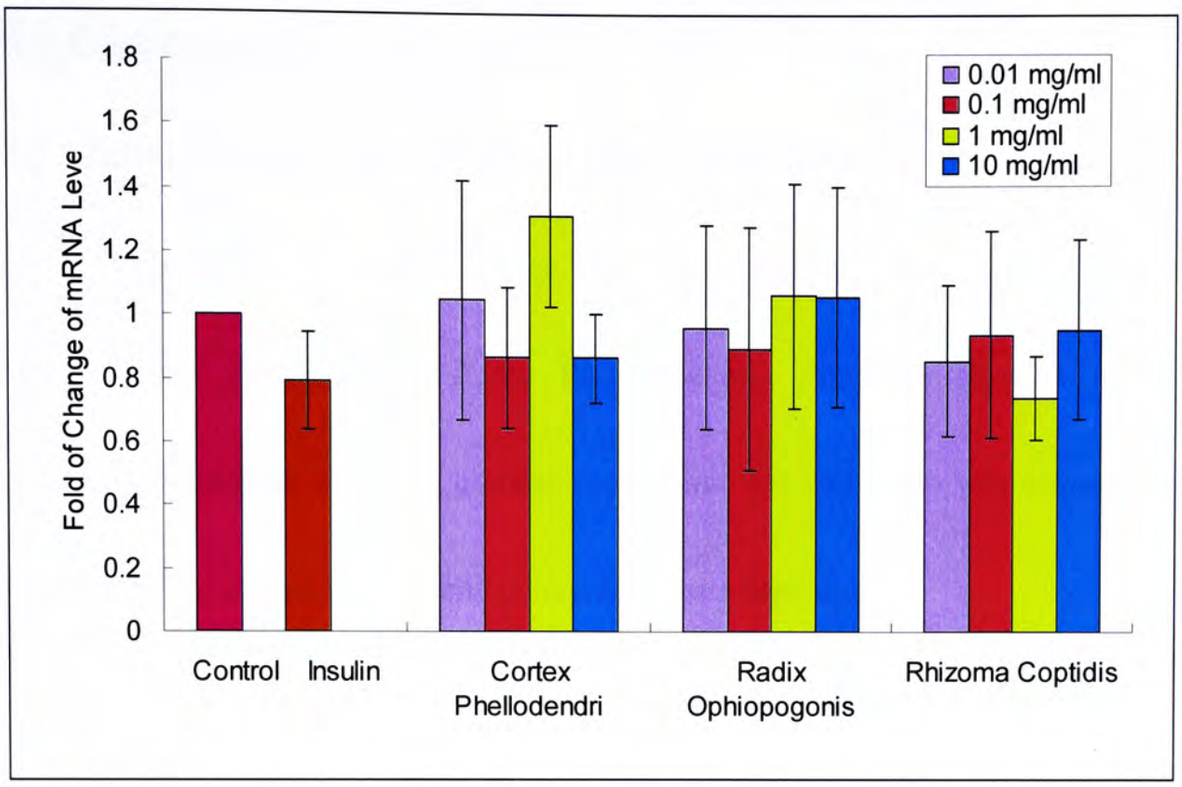


Fig. 3.9 GLUT4 gene expression in 3T3-L1 adipocytes investigated with real-time PCR. 3T3-L1 adipocytes were incubated for 30min with different herbal water extracts, then another 30min with 100mM insulin (for those with insulin treatment groups). Then total RNA was extracted and GLUT4 gene expression was investigated by real-time PCR method. The results were compared with that of the control group without any treatment (100%). Data was expressed as mean ±SD (n=3). There is no significant increase in gene expression among all treatment groups.

3.7 Discussion

3.7.1 Discussion of Hepatic Gluconeogenesis Studies

Among all tested herbs, Cortex Phellodendri (5mg/ml), Rhizoma Copitidis (1mg/ml and 5mg/ml) and Radix Pseudostellaria (5mg/ml) showed the most promising inhibitory effect on glucose production. No cell death was observed after drug treatment at 5mg/ml, which is considered as a high dosage.

Since this assay was to investigate the glucose production effect, any glucose present in the herbal water extract would give false positive results. Moreover, feedback inhibition of glucose production by glucose itself also may have a chance of leading into false positive results. But in H4IIE hepatic glucose production assays, the cells that treated with herbal water extracts was washed thoroughly by PBS solution, before glucose-free glucose production buffer was added, so glucose in the herbal extract was being washed away and would not exert any effect on the assay.

For further confirmation of the inhibitory effect of the potent herbs, PEPCK activity could be studied which measure the consumption of NADH inside the H4IIE cells (Emoto *et al.*, 1993; Wiese *et al.*, 1991). Studies on the gene expression of gluconeogenic enzymes could also be conducted (Waltner-Law *et al.*, 2002; Wang *et al.*, 2000).

3.7.2 Discussion of Intestinal Glucose Absorption Studies

In the BBMV intestinal glucose uptake assay, due to the very small final glucose concentration in the reaction mixture, which was only 0.067mM, any extra glucose present inside herbal extract would reduce the absorption of radiolabeled glucose and result in false positive results. But there is no effective method nowadays used to remove glucose in herbs. Hence, organic extracts of each herb were used instead of water extracts, as glucose could dissolve in water but not in non-polar solvent (the extraction method was discussed previously). In this assay, dichloromethane and hexane extract of each herb was investigated.

The concentration of each treatment used was 1mg/ml, as the herbal extract only dissolved in absolute ethanol, but high ethanol content would affect the result of glucose absorption of BBMV, so the concentration of herbal extract chosen should depend on the final ethanol concentration inside the reaction mixture. Upon several trials (data not shown), 1mg/ml of herbal extract with a final ethanol concentration of 2% v/v was most suitable and acceptable. There was no significant difference between water and 2% ethanol as the solvent used in the reaction mixtures.

Among all tested herbs, dichloromethane extracts of Radix Glycyrrhizae, Radix Ophiopogonis and Radix Trichosanthis showed the most potent and significant inhibitory effect of intestinal glucose absorption; while hexane extracts of Radix

Glycyrrhizae, Radix Ophiopogonis and Radix Trichosanthis exerted the most potent and significant inhibitory effect of intestinal glucose absorption. It suggests that they might contain some potent components on inhibiting SGLT1 in the small intestine.

It is deduced that the herbal extract affect the glucose absorption though SGLT1. Since BBMV is a model without any enzyme or ribosomes (Kessler *et al.*, 1978; Schmitz *et al.*, 1973), further study of the mechanism of how herbal extracts inhibit the BBMV glucose absorption, such as α-glucosidases activity, is impossible (Kim *et al.*, 2004c; Choi *et al.*, 2000).

3.7.3 Discussion of Fibroblast Glucose Uptake Studies

The results showed that most of the herbal extracts (10mg/ml) gave significant stimulation on radiolabelled 2-deoxyglucose uptake into Hs68 fibroblasts through GLUT1. Radix Codonopsis (10mg/ml), Radix Paeoniae Alba (10mg/ml) and Semen Ziziphi Spinosae (0.01mg/ml) showed most potent significant enhancing effect on peripheral tissue glucose uptake. In such a high concentration used, cytotoxicity needs to be considered. For Cortex Phellodendri and Rhizoma Coptidis treatment groups, cell death was observed in 10mg/ml, and led to a decrease in radiolabeled 2-DOG uptake (inhibitory effect showed in the results section). Moreover, as the concentration increased from 0.01mg/ml to 1mg/ml, the level of stimulatory effects of Cortex

Phellodendri and Rhizoma Coptidis on radiolabeled 2-DOG decreased, which illustrated the cytotoxicity effects of these two herbs when use in high dosages.

Although skin fibroblast is not a major insulin responsive tissue, it is believed that the stimulation in the basal glucose uptake through GLUT1 in insulin non-responsive tissues can also lower the blood glucose level (Gherzi *et al.*, 1992).

In this assay, as the incubation time of the herbal extracts was only 30 minutes, which was not likely to be triggered by increasing the expression level of GLUT1 mRNA, but by GLUT1 transloaction (Bosch *et al.*, 2004; Powell *et al.*, 1999), and it was necessary to be studied further.

3.7.4 Discussion of Adipocyte Glucose Uptake Studies

3T3-L1 adipocytes were greatly expressed with GLUT4 which is responsible for insulin sensitive glucose uptake. The three herbs chosen from the previous *in vitro* screening with most potent anti-diabetic effects were carried out this assay. In this cell model, 10nM of insulin was added during the experiment to mimic the type 2 diabetes condition, and for studying the improvement effect of herbal extract on insulin sensitivity (Fukuen *et al.*, 2005; Powell *et al.*, 1999). Moreover, the herb-insulin interaction could be studied by the insulin-stimulated glucose uptake in the 3T3-L1 adipocytes (Kamei *et al.*, 2002; Nugent *et al.*, 2001).

From the results, the radiolabeled 2-DOG uptake of insulin treated positive control group was higher than that of non-insulin treated negative control group. This proved that insulin had the stimulatory effect on glucose uptake of adipocytes. Cortex Phellodendri (10mg/ml) exerted the interaction with insulin to enhance the glucose uptake into adipocytes, and showed to have improvement in insulin sensitivity in the cells. On the other hand, for the other herbal treatment groups, they suppressed the glucose uptake.

The enhancing effect of such glucose uptake may be achieved by glucose transporters. In the 3T3-L1 adipocytes, GLUT1 and GLUT4 are expressed. In the next section, the gene expression of GLUT4 upon stimulation of herbal water extracts and its relationship on glucose uptake will be discussed.

In future studies, GLUT4-GFP (green fluorescence protein) can be used to study the glucose uptake mechanism through GLUT4 transloaction after herbal extract treatment (Powell *et al.*, 1999). The herbal extracts may possess insulin-like effects on the 3T3-L1 adipocytes and stimulate the translocation of the GLUT4 proteins from the intracellular vesicles to the plasma membrane (Harrison *et al.*, 1992). GLUT1 also has translocation but to a smaller extent (Calderhead *et al.*, 1990), and this effect has been demonstrated in Hs68 fibroblast glucose uptake study described in section 3.4.

3.7.5 Discussion of Glucose Transporter Type 4 (GLUT4) Expression Studies

Insulin-stimulated glucose uptake in adipocytes is highly correlated with the expression level of GLUT4, and altered expression of GLUT4 may play a role in the insulin resistance of type 2 diabetes (Shepherd and Kahn, 1999). Because of this, it is interesting to identify whether traditional Chinese herbs could affect the GLUT4 regulations in adipocytes with insulin-like effect (Nugent *et al.*, 2001).

As there was no significant increase in GLUT4 gene expression after 30 minutes herbal water extract treatment from RT-PCR and also real-time RT-PCR, that illustrated that there was no increase in the level of GLUT4 gene after 30 minutes incubation with different herbal treatments.

This disappointing result might be due to insufficient herbal treatment incubation time, as the translational activity of glucose transporters usually need to take more than two hours (McGowan *et al.*, 1995). Hence, we can conclude that the 3T3-LI adipocytes glucose uptake assay was not affected by GLUT4 gene expression within 30 minutes.

According to the results, hypothesis could be made that the glucose uptake in was due to an instant stimulation of GLUT4 uptake ability or other glucose uptake mechanisms.

To further investigate, longer incubation time of herbal extracts on the cells is needed for GLUT4 gene translation.

3.7.6 Conclusion

The results of *in vitro* studies were summarized in Table 3.1. For BBMV glucose uptake assay, H4IIE hepatic gluconeogenesis assay, inhibitory effects were desirable for anti-diabetic agents; while for the Hs68 fibroblast glucose uptake and 3T3-L1 adipocyte glucose uptake assays, stimulatory effects were desirable.

Among 14 herbs tested, three herbs were selected out for further studies in diabetic animal model to confirm their potent anti-diabetic effects. Cortex Phellodendri, which has excellent inhibition in hepatic glucose production and desirable effect on intestinal glucose absorption and fibroblast/adipocyte glucose uptake, was chosen. Rhizoma Corptidis, which showed all positive effect in anti-diabetic with lower dosage then other herbs with positive result, was suitable for further investigation. Finally for Radix Ophiopogonis, although it had negative result in ability of inhibiting gluconeogenesis, its strong inhibitory effect on intestinal glucose absorption was desirable for further study in animal model.

The advantage of using *in vitro* models compared with *in vivo* models and other clinical studies for pharmacological investigation that they are more convenient to

tackle with, they give fast response and hence faster to have results and allow the possible way to conduct large scale experiments. It also allows specific pharmacological mechanistic studies to be made, and there are no need to use so many animal, and thus easier for ethnical approval.

The disadvantages of using *in vitro* assays are: First, the results obtained only represent the effect on specific types of cells, while any effect on animals or on humans is still not yet known, hence the results should be further confirmed by *in vivo* and clinical studies; second, cells chosen to carry out experiments from other species sometimes may not be human cells, the positive results using cell types could not represent the actual effect inside humans; third, since one *in vitro* study could only focus one specific mechanism, it could not cover other possible pharmacological mechanisms, hence more *in vitro* models are required.

As restoring insulin secretion in pancreatic β-cells can be one of the modes of action in controlling blood glucose level of type 2 diabetic patients. In future studies, insulin-secreting cell lines, such as the hamster HIT-T15 β-cells cell line and RIN-m5f can be used to study the enhancing effect on insulin secretion by traditional Chinese herbs (Noor *et al.*, 1989)

Chinese Herbs	H4IIE	BBMV (CH_2Cl_2)	BBMV (Hexane)	Hs68	3T3-L1
Radix Paeoniae Alba	+27.51 (0.2)	-31.42 ***	-78.38 ***	+114.11 (10)	/
Semen Platycladi	-17.30 (5)	-78.18 ***	-57.95 ***	+90.28 (10) **	/
Radix Codonopsis	+8.00 (1)	-31.66 ***	-35.16 ***	+133.86 (10) *	/
Herba Lophatheri	+7.98 (5)	-45.06 ***	-54.01 ***	-3.02 (0.1)	/
Radix Glycyrrhizae	-27.96 (5)	-82.76 ***	-77.50 ***	+41.22 (1)	/
Radix Puerariae	+29.46 (5)	-44.59 ***	-35.57 ***	+5.05 (1)	/
Ramulus Cinnamorni	-15.82 (1)	-70.50 ***	-77.67 ***	+52.57 (0.01)	/
Cortex Phellodendri	-81.55 (5) **	-23.50 *	-69.48 ***	+58.19 (0.1) *	+33.32 (+insulin) (10) +63.44 (-insulin) (1)
Rhizoma Copitidis	-64.01 (1) **	-28.63 **	-58.13 ***	+58.19 (0.01)	-35.28 (+insulin) (1) * +79.03 (-insulin) (0.1)
Radix Ophiopogonis	+9.22 (0.2)	-93.10 ***	-98.13 ***	+50.45 (0.1)	-26.11 (+insulin) (0.01) -3.81 (-insulin) (0.1)
Radix Trichosanthis	-41.95 (5)	-81.23 ***	-95.43 ***	+18.95 (1)	/
Radix Pseudostellariae	-88.80 (5) **	-14.24 *	-63.02 ***	+42.80 (1) **	/
Rhizoma Anemarrhenae	+19.06 (0.2)	-79.99 ***	-71.58 ***	+39.09 (0.1)	/
Semen Ziziphi Spinosae	-3.39 (1)	-4.30	-75.60 ***	+202.37 (0.01) **	/

Table 3.1 Summary of *in vitro* studies of fourteen selected traditional Chinese herbs. The number blanketed represents the concentration in mg/ml aqueous extract, except in BBMV studies with hexane or dichloromethane extracts. Significantly different from control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Chapter 4 Purification of Cortex Moutan

4.1 Introduction

With the potent *in vitro* inhibition of BBMV glucose uptake, bioassay-guided fractionation of Cortex Moutan was carried out in order to find the active constituent(s).

4.1.1 Phytochemical Studies of Cortex Moutan

Cortex Moutan was a commonly used traditional Chinese herb. The major active components inside are divided into five groups:

1. Acetophenones:

   Paeonol (Fig. 4.1a(i)) is the major phenolic acetophenone inside Cortex Moutan, and some six others minor acetophenones such as 2,5-dihydroxy-4-methoxyacetophenone (Fig. 4.1a(ii)), acetovanillone (Fig. 4.1a(iii)), acetoisovanillone (Fig. 4.1a(iv)), 2,3-dihydroxy-4-methoxyacetophenone (Fig. 4.1a(v)), resacetophenone and 2,5-dihydroxy-4-methylacetophenone (Fig. 4.1a(vi)), they exhibited potent antibacterial, anti-inflammatory and anticoagulation activities (Lin and Chern, 1991; Lin *et al.*, 1999).

2. Terpenoids:

   Terpenoids including monoterpenes such as paeonisuffrone (Fig. 4.1b(i)), deoxypaeonisuffrone (Fig. 4.1b(ii)), paeonisuffral (Fig. 4.1c), isopaeonisuffral and paeonisothujone (Yoshikawa *et al.*, 1994; Yoshikawa *et al.*, 2000); and also riterpenoid, mudanpinoic acid A (Fig. 4.1d), were found in Cortex Moutan, which have analgesic, sedative and anti-inflammatory effects (Lin *et al.*, 1998; Lin *et al.*, 1996).

3. Glycosides:

Several paeonol glycosides are found in Cortex Moutan, including paeonoside (Fig. 4.1a(vii)), apiopaeonoside, paeonolide (Fig 4.1e), and different suffruticosides (Fig. 4.1e(ii, iii, iv)) (Matsuda *et al.*, 2001). There are also monoterpene glycosides, including paeoniflorin (Fig. 4.1f(i)), oxypaeoniflorin Fig. 4.1f(ii)), mudanpioside A-F, and their benzoyl or galloyl derivatives, and many mudanpiosides (Matsuda *et al.*, 2001; Ding *et al.*, 1999; Yoshikawa *et al.*, 2000; Lin *et al.*, 1996). Alpha-benzoyloxypaeoniflorin has anti-oxidative effect (Ryu *et al.*, 2001). Paeoniflorin was reported for its anti-diabetic effect, by reducing basal glycaemia and improving glucose tolerance of STZ-treated rats (Hsu *et al.*, 1997).

4. Tannins:

1,2,3,4,6-penta-O-galloyl-β-D-glucose (Fig. 4.1g) is one of the galloyl-glucose with anti-cancer effect (Wu *et al.*, 2003b).

5. Polysaccharides:

One of the polysaccharides called polysaccharide-2b which is present in Cortex Moutan has been shown to have hypoglycaemic activity in a diabetic mouse model(Hong *et al.*, 2003).

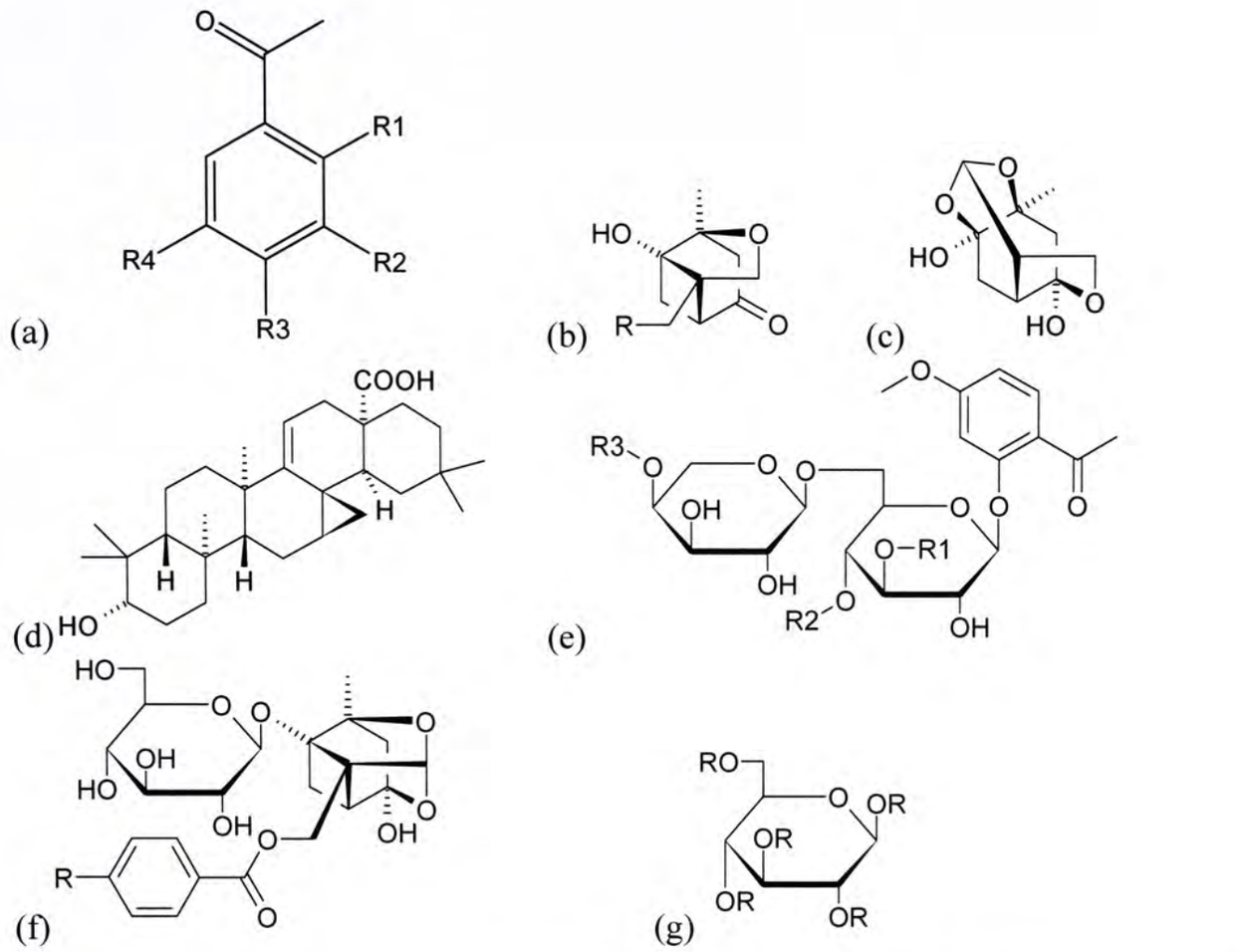


Fig. 4.1 Structures of compounds found in Cortex Moutan

(a) i. Paeonol: R1 = OH, R2 = H; R3 = OCH_3, R4 = H;
ii. 2,5-dihydroxy-4-methoxyacetophenone: R1 = OH, R2 = H, R3 = OCH_3, R4 = OH;
iii. Acetovanillone: R1 = H, R2 = CH_3, R3 = OH, R4 = H;
iv. Acetoisovanillone: R1 = H, R2 = OH, R3 = CH_3, R4 = H;
v. 2,3-dihydroxy-4-methoxyacetophenone: R1 = OH, R2 = OH, R3 = OCH_3, R4 = H;
vi. 2,5-dihydroxy-4-methylacetophenone: R1 = OH, R2 = H, R3 = CH_3, R4 = OH;
vii. Paeonoside: R = β-D-glucopyranosyl;
(b) i. Paeonisuffrone: R = OH; ii. Deoxypaeonisuffrone: R = H;
(c) Paeonisuffral; (d) Mudanpinoic acid A
(e) i. Paeonolide: R1 = R2 = R3 = H;
ii. Suffruticoside C: R1 = R2 = H, R3 = galloyl
iii. Suffruticoside D: R1 = R3 = H, R2 = galloyl;
iv. Suffruticoside E: R1 = β-D-glucopyranosyl, R2 = R3 = H;
(f) i. Paeoniflorin: R = H; ii. Oxypaeoniflorin: R = OH;
(g) 1,2,3,4,6-Penta-O-galloyl-β-D-glucose: R = galloyl

4.2 Organic Extraction of Cortex Moutan

4.2.1 Extraction Material and Methods

One and a half kilograms of raw material of Cortex Moutan were cut into small pieces and covered with 2.5L of 95% EtOH (EtOH-H_2O, 95:5, v/v; Uni-Chem, South Kearny, NJ, USA). After refluxing for two hours, the extract solution was collected and fresh 95% EtOH was added for another two-hour extraction. Two extractions were combined, and the solvent was evaporated using a rotary evaporator to obtain the ethanolic extract of Cortex Moutan (CM-A).

CM-A was re-dissolved in 500ml distilled water and partitioned with 500ml hexane (Lab-Scan, Bangkok, Thailand). Hexane fraction was collected, and the water fraction was partitioned again with 500ml of fresh hexane. Two hexane fractions were combined. Excess anhydrous sodium sulphate (MERCK, Darmstadt, Germany) was added to absorb remaining moisture in hexane extract. The mixture was filtered and the hexane extract (CM-C) was dried using a rotary evaporator. The remaining water fraction (CM-B) was further partitioned with 500ml dichloromethane (Lab-Scan, Bangkok, Thailand). Dichloromethane fraction was collected, and the water fraction was again partitioned with another 500ml of fresh dichloromethane. Two dichloromethane fractions were combined and the moisture inside was absorbed by excess anhydrous sodium sulphate. The mixture was filtered and the dichloromethane extract (CM-D) was dried using a rotary evaporator. The water fraction (CM-E) was discarded. A simplified extraction procedure was shown in Fig. 4.2.

4.2.2. Results

The Cortex Moutan raw material was extracted by 95% EtOH, giving the ethanolic extract of Cortex Moutan (CM-A), with extraction yield of 11.68% w/w. CM-A was

partitioned between water and hexane. The extraction yields of the hexane fraction (CM-C) and dichloromethane fraction (CM-D) from the raw material were 1.33% w/w and 1.16% w/w respectively. CM-C was a pale brown solid, and CM-D was yellowish brown solid, both with a characteristic odour of raw Cortex Moutan.

The purification of active component(s) was bio-assay guided according to BBMV assay results. As in BBMV assay, any glucose present in the herbal extracts could affect the accuracy of the results, hence, non-polar organic solvent was used in extraction and partition in order to get rid of undesirable glucose or other sugars. Thus, both CM-B and CM-E water fractions were discarded as they might contain glucose which are extracted from the herbal extracts.

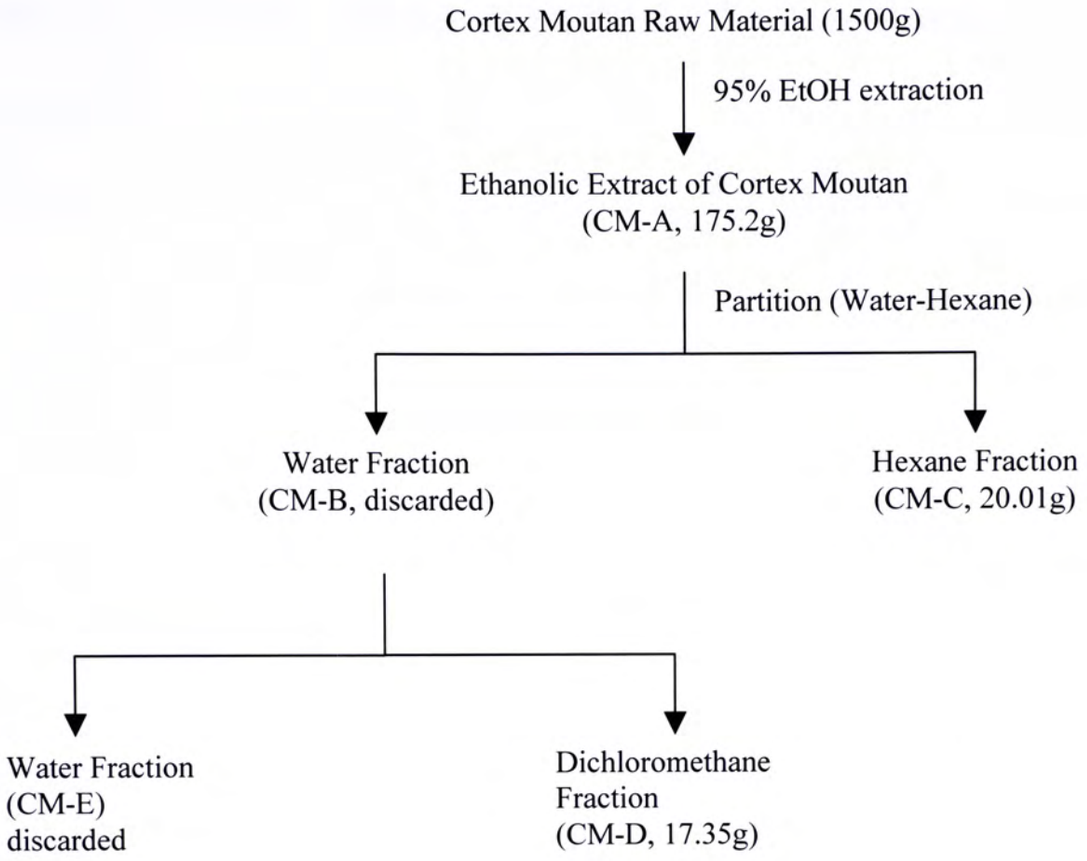


Fig. 4.2 Organic Extraction Procedure of Cortex Moutan

4.3 BBMV Glucose Uptake Assay with Cortex Moutan Organic Extract (CM-C and CM-D)

The anti-diabetic activity of CM-C was confirmed by another student before (data not shown) (Lau, 2004). The activity CM-D on BBMV intestinal glucose absorption compared with CM-C was studied in order to find out the active component(s) inside Cortex Moutan.

4.3.1 Material and Methods

The materials and methods were the same as described in section 3.2.2. As both CM-C and CM-D fractions were water insoluble, they were re-dissolved in 100% EtOH (MERCK, Darmstadt, Germany), and diluted by water to become 4% v/v EtOH, and the final concentration would become 2% v/v EtOH in the reaction mixture. The concentration of herbal extract was 2mg/ml and the final concentration was 1mg/ml in reaction mixture. The result of each treatment was compared against 2% v/v EtOH treatment (as control). Water control was performed to monitor the effect of 2% v/v EtOH on glucose uptake of BBMV.

4.3.2 Results

The results of BBMV glucose uptake were shown in Fig. 4.3 As proven before, no significant difference was found between 2% v/v EtOH group and the water control group, therefore 2% v/v EtOH would not affect the glucose uptake of BBMV and could be used as a control. The dosage used for both CM-C and CM-D were 1 mg/ml, and they both significantly inhibited glucose absorption of BBMV.

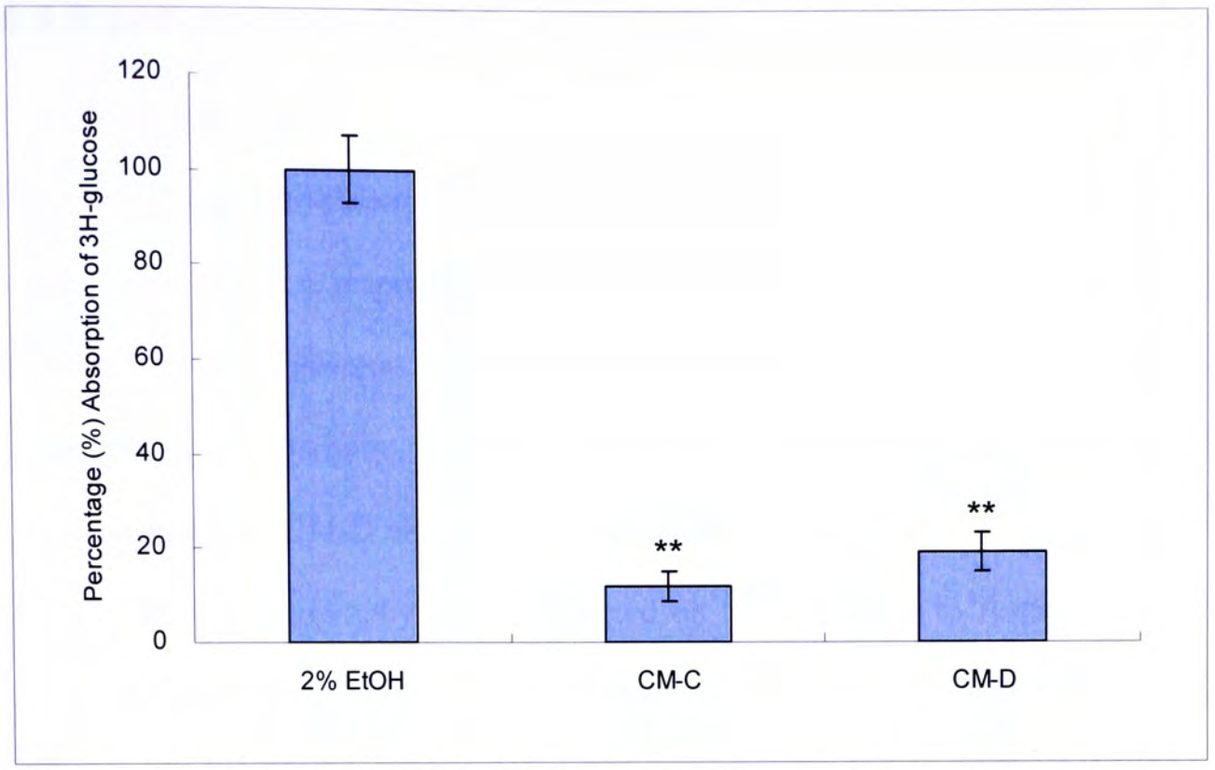


Fig. 4.3 Effects of CM-C and CM-D fractions on glucose absorption of BBMV. 2% EtOH served as control. The results are expressed as mean±SD, n=12. Asteristo were used to indicate the significantly difference from 2% v/v EtOH: *p<0.05, **p<0.01, ***p<0.001.

4.4 Fractionation of CM-C and CM-D

CM-C was fractionated by another student and the pure compound paeonol was proven to have anti-diabetic effects *in vitro* (Lau, 2004). But there may be other compound(s) which is responsible for the anti-diabetic effect since the inhibitory effect in BBMV assay of paeonol was not as strong as CM-C, so there must be other active component(s). Furthermore, as shown in the BBMV intestinal glucose absorption assay in the last section, CM-D also significantly inhibited glucose absorption, hence, silica-gel column-chromatography were conducted on both fractions to further find out the active components. The fractionation was guided by BBMV glucose uptake assay. Using column-chromatography allowed separation of compounds in a large scale, providing adequate amounts of samples for the bioassay and structure elucidation.

4.4.1 Material and Methods

4.4.1.1 Chemicals

Here is the list of the chemicals used in this experiment:

1. Purchased from MERCK (Darmstadt, Germany):

   Silica-gel 60, anisaldehyde, glacial acetic acid, and silica-gel 60 F_{254} pre-coated TLC plate.

2. Purchased from Lab-Scan (Bangkok, Thailand):

   Methanol (MeOH), hexane and ethyl acetate (EtOAc).

3. Purchased from BDH Laboratory Supplies (Poole, UK)

   Concentrated sulphuric acid (H_2SO_4).

4.4.1.2 Methods

Fractions CM-C (6g) and CM-D (6g) were completely dissolved in MeOH, and

mixed with 10g silica-gel 60 respectively. The solvent was completely dried by rotary-evaporator to obtain the mixtures of CM-C/silica gel and CM-D/silica gel in powder form.

One hundred and fifty grams of silica-gel 60 were mixed with the eluent (hexane-EtOAC, 7:3, v/v). The mixture was loaded in a glass column and until the silica gel was tightly packed inside the column. The length of column was 400mm and the diameter was 320mm. CM-C/silica gel or CM-D/silica gel mixture was gently placed on top of the column. The column was then eluted with the eluent under gravity. Fractions were collected by a fraction collector and the fractions collected were pooled together according to the chemical profile of fractions by thin layer chromatography analysis. Finally the column was flushed with 100% EtOAc in order to elute all the remaining substances inside the column. Fractions were completely dried with a rotary evaporator and weighed.

For TLC analysis, silica gel 60 F_{254} on aluminum sheets was used as the stationary phase, and the mobile phase components were same as the eluent of silica gel column chromatography (i.e. hexane-EtOAC, 7:3, v/v). For each fraction, samples were applied onto a TLC plate at the baseline using a capillary tube, with 1cm space from the bottom edge of the plate. 10ml of mobile phase was added to a covered glass chamber. After the chamber was pre-equilibrated with the mobile phase for about 5 minutes, the TLC plate was placed inside the chamber, and it was then removed when the solvent front of mobile phase was about 1cm below the top edge of the plate. The plate was dried, and visualized under ultraviolet light (UV), and/or after spraying with different reagent: (1) anisaldehyde-sulphuric acid reagent (0.5ml anisaldehyde, 50ml acetic acid, 1ml sulphuric acid, freshly prepared) and heating at 100°C for five minutes; or (2) 10% v/v H_2SO_4 in EtOH,and heating at 110°C for 5 minutes.

The R_f value was determined and the colour of each spot on TLC plate was then recorded. The calculation of R_f value is shown below:

$$R_f \text{ value} = \frac{\text{Distance from baseline to centre of spot}}{\text{Distance from baseline to solvent front}}$$

4.4.2 Results

Both CM-C and CM-D were fractionated using silica-gel column chromatography.

For CM-C, a total of 98 fractions were obtained, and combined into final 12 fractions according to their chemical profiles, known as CM-C1 to CM-C12. The weights of fractions were shown in Table 4.1. The heaviest fraction CM-C2 (4.35g) was proven to be paeonol by another student (Lau, 2004), whereas for the other fractions, the yield was very low. The total yield of all CM-C sub-fractions was 85.22%, the relative high yield showed that most of the components had been collected and the amount of substance left inside the column was small.

For CM-D, a total of 95 fractions were obtained, and they were combined into final 6 fractions according to their chemical profiles, known as CM-D1 to CM-D6. The weights of fractions are shown in Table 4.2 The heaviest fraction CM-D1 (4.56g) was proven to be paeonol by another student (Lau, 2004), for the other fractions, the yield was very low. The total yield of all CM-C sub-fractions was 76.67%, which also showed that most of the components had been eluted out.

The TLC chromatogram of CM fractions was shown in Fig. 4.4. The same amount of fraction solution was applied onto the TLC plate. It revealed that most of the components inside both fractions were the same. The yellow spot with R_f value of 0.51 is paeonol.

In Fig. 4.5, the whole profile of CM-C was shown. Obvious polarity gradient was

observed in CM-C profile. There are another 2 yellow spots present in CM-C7 and CM-C8, which were not clearly seen in Fig. 4.4. It might be due to a small amount present in the Cortex Moutan hexane fraction. In Fig. 4.6, CM-D profile was shown. An obvious polarity gradient was observed too. CM-D1 was proved to be paeonol, and CM-D3 to CM-D6 were all yellow in colour in TLC chromatogram. But there was only one yellow spot (except paeonol one) in CM-D, hence, further fractionation was needed.

CM-C Fraction	Weight (mg)	Yield (%)
1	101.1	1.68
2	4335.3	72.26
3	32.8	0.54
4	96.1	1.60
5	148.8	2.48
6	55.4	0.92
7	30.2	0.50
8	16.9	0.28
9	15.5	0.26
10	8.8	0.15
11	8.5	0.14
12	103.7	1.73
Total	4953.1	82.55

Table 4.1 The table showing the yield of CM-C sub-fractions

CM-D Fraction	Weight (mg)	Yield (%)
1	4560.0	76.00
2	7.8	0.13
3	8.0	0.13
4	10.3	0.17
5	6.8	0.11
6	7.3	0.12
Total	4600.2	76.67

Table 4.2 The table showing the yield of CM-D sub-fractions

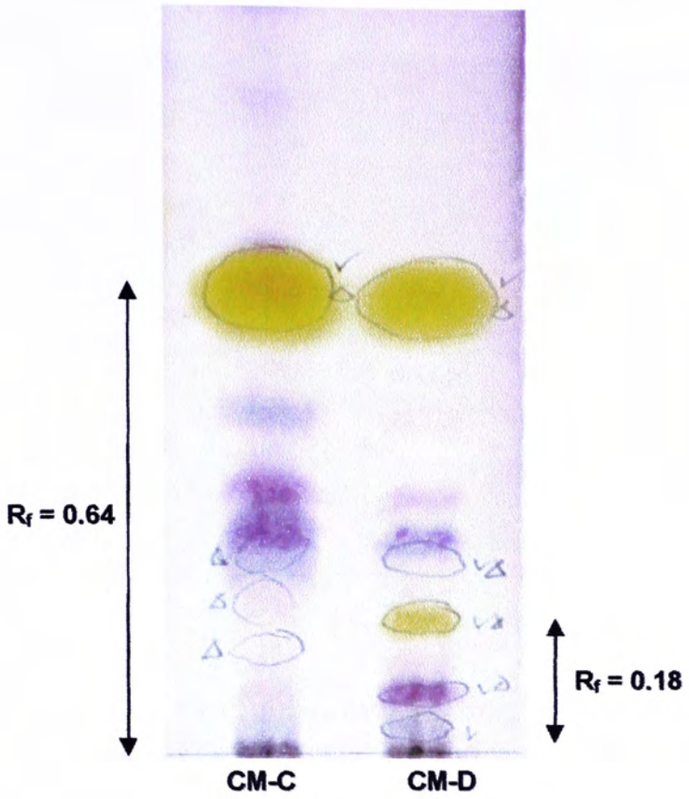


Fig. 4.4 The TLC chromatogram of CM-C and CM-D.

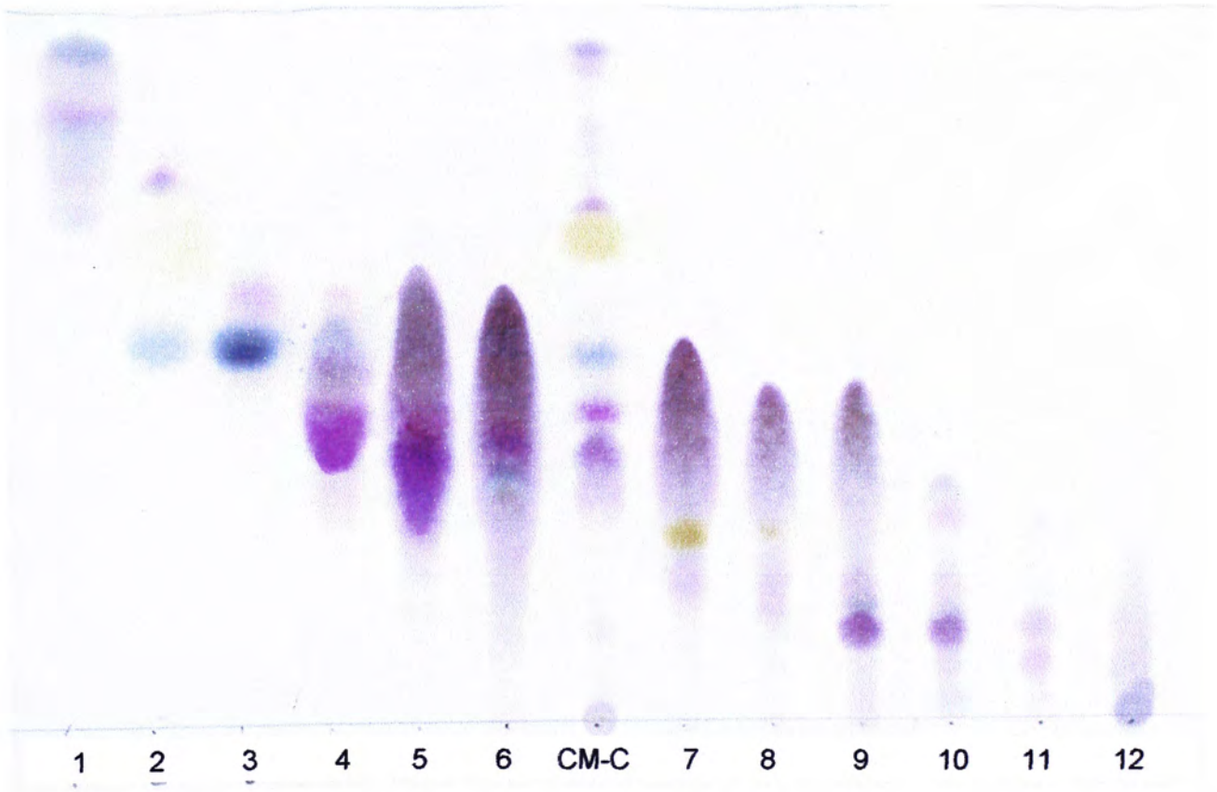


Fig. 4.5 The TLC chromatogram of CM-C1 to 12.

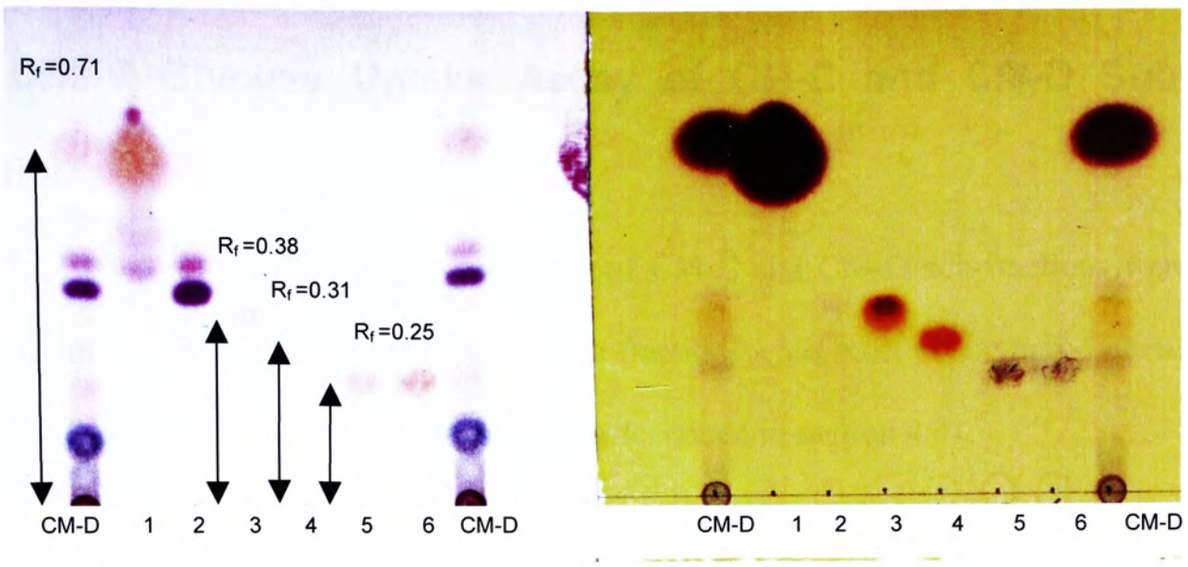


Fig. 4.6 The TLC chromatogram of CM-D1 to CM-D6. Left: TLC chromatogram sprayed with anisaldehyde-sulphuric acid reagent. Right: TLC chromatogram sprayed with 10% v/v H_2SO_4 in EtOH, brown colour indicated that those were phenoic compounds.

4.5 BBMV Glucose Uptake Assay of CM-C and CM-D Sub-fractions

The BBMV intestinal glucose absorption of CM-C and CM-D sub-fractions were tested in this section in order to find out which fraction(s) has most potent anti-diabetic effect. All the steps of assay were performed as described in section 4.31.

4.5.1 Results

BBMV glucose uptake assay was performed with the CM-C and CM-D sub-fractions and results were shown in Fig. 4.7 and 4.8. All fractions of CM-C and CM-D (except CM-D2) showed significant inhibition on BBMV intestinal glucose absorption. The most potent result in this assay in CM-C sub-fractions was CM-C8, which suppressed the glucose absorption into BBMV by 66.6%; while that of CM-D sub-fractions was CM-D3, which suppressed by 69.0%.

The BBMV results were compared with the TLC chromatogram (Fig. 4.5 and 4.6). It was found that pale yellow spots with R_f value around 0.26 was found in fraction CM-C8 and that with R_f values around 0.38, 0.31 and 0.25 were found in fractions CM-D3 to CM-D5. So it was suspected that the yellow spot should be another active component in Cortex Moutan.

According to Fig. 4.4, as nearly same amount of sample solutions were applied onto the TLC plate, hence the amount of each fraction was revealed. The amount of yellow spot in CM-C was much less than CM-D. Hence, further investigation was done with the CM-D fraction rather than CM-C in order to find out the active component(s) other than paeonol (CM-C2 or CM-D1).

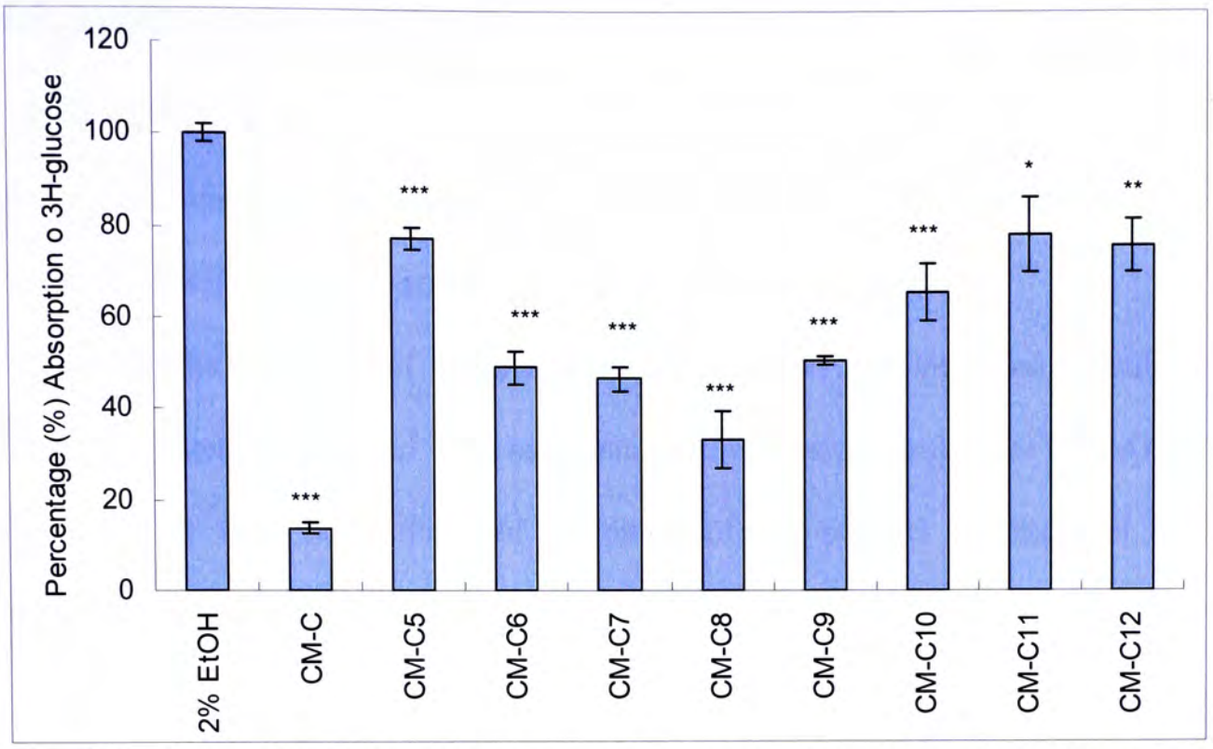


Fig. 4.7 Effects of CM-C sub-fractions on glucose absorption of BBMV. 2% EtOH served as control. The results are expressed as mean±SD, n=12. Asteristo were used to indicate the significantly difference from 2% v/v EtOH: *p<0.05, **p<0.01, ***p<0.001.

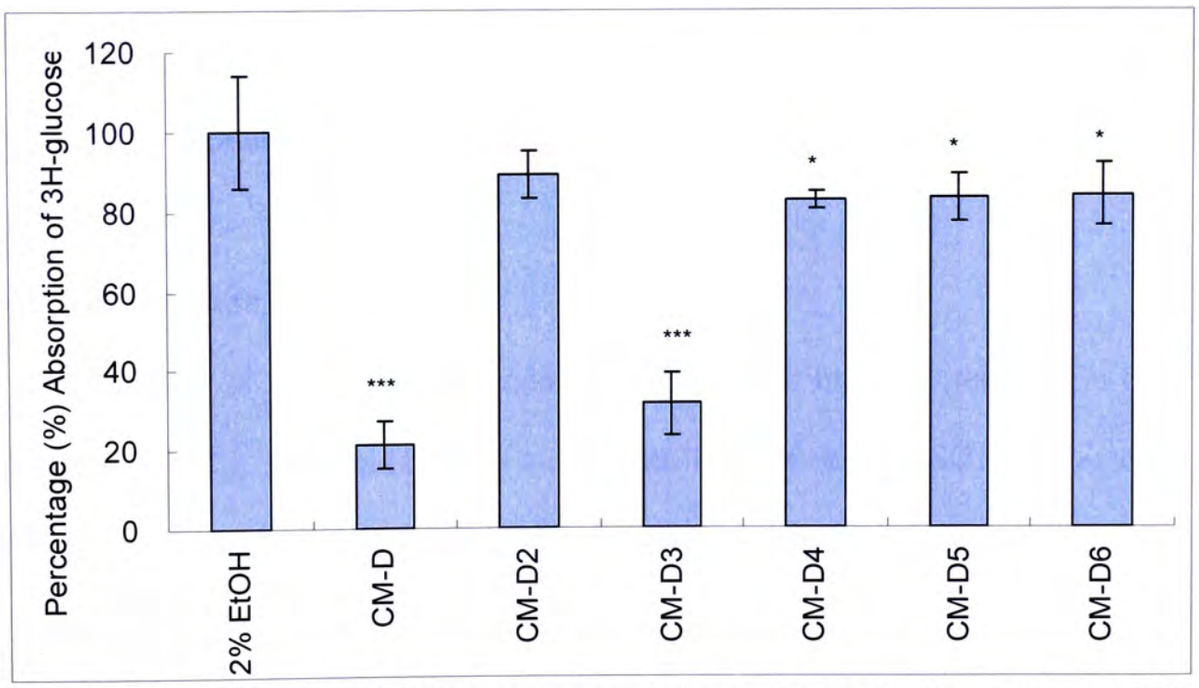


Fig. 4.8 Effects of CM-D sub-fractions on intestinal glucose absorption of BBMV. 2% EtOH served as control. The results are expressed as mean±SD, n=12. Asteristo were used to indicate the significantly difference from 2% v/v EtOH: *p<0.05, **p<0.01, ***p<0.001.

4.6 Sulfonylation of CM-D1

CM-D1 (paeonol), as said before, was proven to have anti-diabetic effect in BBMV *in vitro*. Although it is present in large abundance in Cortex Moutan and has powerful anti-diabetic effect, it has a disadvantage in that it is not water soluble.

Hence, the solubility of organic compound paeonol was increased by sulfonaton (Wang and Tang, 1983), and ionic compound sodium paeonol sulfonate (CM-D1s) was formed. And then the activity of inhibition of the product in intestinal glucose absorption was investigated.

4.6.1 Material and Methods

4.6.1.1 Chemicals

1. Purchased from Sigma (St. Lousi, MO, USA)

   Paeonol (2-hydroxy-4-methoxyacetopheone), sodium chloride (NaCl)

2. Purchased from Lab-Scan (Bangkok, Thailand)

   Ethyl acetate (EtOAc), Concentrated sulphuric acid (H_2SO_4)

4.6.1.2 Methods

Sulfonation of paeonol was carried out according to literature record (Hu G, 2004; Wu *et al.*, 2003a). Paeonol (5g) was mixed with 15ml of conc. H_2SO_4 in a round bottom flask. The mixture was heated at 75°C inside a water bath for 25 minutes with continuous stirring. After that, the solution was slowly poured into 500ml distilled water. The acidic solution was then extracted with equal amounts of EtOAc for three times, and a small amount of active charcoal was added with warming in order to remove the colour of the acidic solution. The solution was filtered and 23% (w/v) of NaCl was added inside, until all NaCl was dissolved totally, then it was cooled down and

crystallized at 4°C. The crystal formed was filtered and dried. The form of the crystal was recorded, and the melting point and its solubility was tested.

4.6.2 Structure Elucidation of CM-D1s

The structure of CD-D1s was elucidated by proton nuclear magnetic resonance (^{1}H-NMR), and compared with reference spectra and previous reports. As the melting point of CM-D1s was too high for mass spectrometry, only ^{1}H-NMR was done. ^{1}H-NMR provided information on the assignment of hydrogen atoms in the studied molecule.

4.6.2.1 ^{1}H-NMR Analysis

^{1}H-NMR analysis was performed using Bruker (Rheinstetten, Germany) Avance DPX300 NMR Spectrometer. Ten milligrams of CM-D1s was dissolved in 0.75ml of deuterium methanol (MeOD; Aldrich, St. Louis, MO, USA). The 300MHz ^{1}H-NMR spectrum was detected. Chemical shifts were detected relative to reference tetramethylsilane.

4.6.3 BBMV Glucose Uptake Assay of CM-D1s

The BBMV glucose absorption of CM-D1s was tested to find out its anti-diabetic ability of CM-D1 after adding sulfonyl group onto the compound.

4.6.4 Results

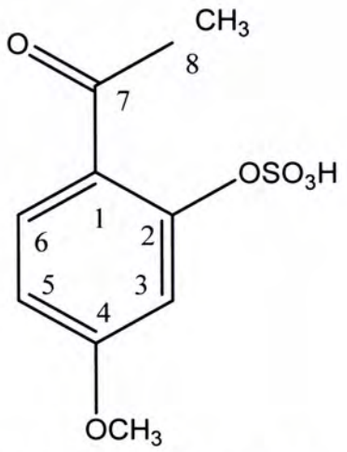
The product (CM-C1s) collected was white needle shape crystal, with 100% yield (1.02g). The melting point of the product was over 200°C and so could not be detected. But it was previously reported to be over 320°C (Wang and Tang, 1983).

Hence mass spectrometry could not be conducted as it could not be vaporized by mass spectrometer.

The solubility was increased after sulfonation of paeonol, which give a water soluble product. The solution was pale yellow.

The structure of sodium paeonol was shown in Fig. 4.9. The structure was deduced from ^{1}H-NMR spectrum which shown in Appendix 2.

Fig. 4.10 showed that all concentrations of CM-D1s (0.25, 0.5 and 1 mg/ml) did not have significant inhibition on BBMV glucose absorption, while paeonol had significant inhibition compared with 2% EtOH control. It proved that although sulfonyl group was added, it did not exert any anti-diabetic effect as shown previously.



Sodium Paeonol Sulfonate
(2-sulfooxy-4-methoxyacetophenone)

Fig 4.9 The structure of sodium paeonol sulfonate.

	Chemical Shift (δ ppm)	
Protons	**CM-D1**	**CM-D1s**
C2		
C3	6.42,s	6.48,s
C5	6.45, d, *J*=7.5	6.48, d, *J*=7.5
C6	7.63,d,*J*=7.5	8.28,d,*J*=7.5
C8	2.56,s	2.51,s
OCH3	3.84,s	3,86,s
CH3		
OSO_3H		3.23,m

Table 4.3 Proton assignments of CM-D1 and CM-D1s.
s – Singlet; d-Doublet; m – Multiplet; *J* – Coupling constant.

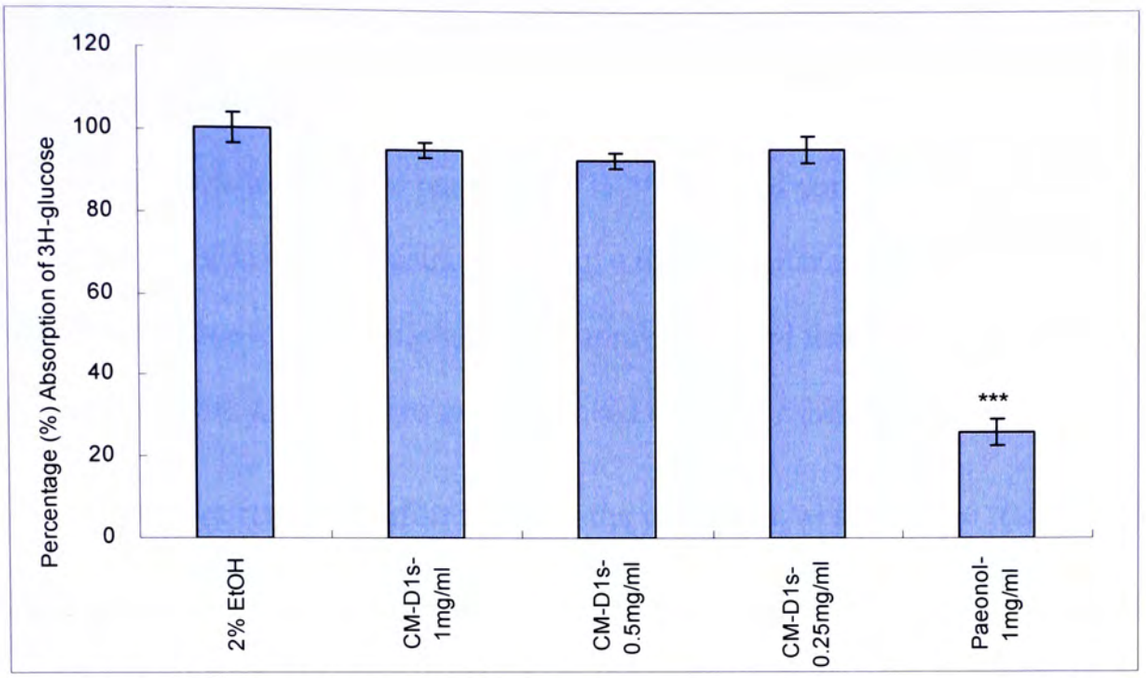


Fig. 4.10 Effects of of CM-D1s and paeonol (CM-D1) on intestinal glucose absorption of BBMV. 2% EtOH served as control. The results are expressed as mean±SD, n=12. Asteristo were used to indicate the significantly difference from 2% v/v EtOH: ***$p<0.001$.

4.7 Structural Elucidation of CM-D3, CM-D4 and CM-D5

Structure of CM-D3, CM-D4 and CM-D5 were elucidated by mass spectrometry (MS) and proton nuclear magnetic resonance (^{1}H-NMR), and compared with reference spectra and previous reports. As illustrated in Fig. 4.6, the similar colour in TLC chromatography which turned brown after sulphuric acid spray indicated that they might be the same type of chemical with a phenol group, so we need to clarifiy their structures.

MS studies provided information on the molecular weight of the tested compounds, and that of the fragments generated during electron impact (EI). ^{1}H-NMR provided information on the assignment of hydrogen atoms in the studied molecule.

4.7.1 Material and Methods

4.7.1.1 Mass Spectrometry

Thermo (Bremen, Germany) MAT 95XL Gas Chromatographic Mass Spectrometer was used in MS analysis. One milligram of sample dissolved in 1μl MeOH was injected to the machine. The low resolution EI spectra were obtained for the analysis of compound fragments and determination of the molecular weights of the compounds.

4.7.1.2 ^{1}H-NMR Analysis

^{1}H-NMR analysis was performed using Bruker (Rheinstetten, Germany) Avance DPX300 NMR Spectrometer. Each of the compounds (7 mg) was individually dissolved into 0.75ml of deuterium chloroform ($CDCl_3$; Aldrich, St. Louis, MO, USA). The ^{1}H-NMR spectra were detected at 300MHz. Chemical shifts were detected relative to reference tetramethylsilane.

4.7.2 Results

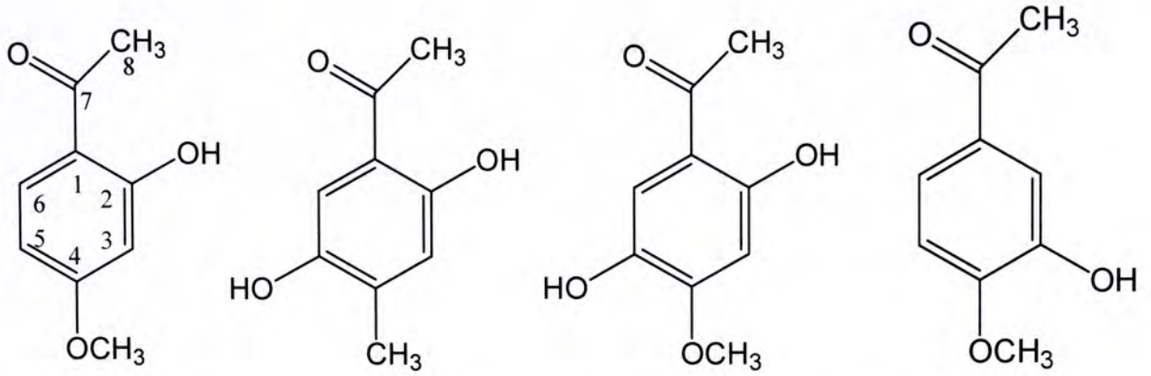
The low resolution EI spectra of CM-D3, CM-D4 and CM-D5 were shown in Appendices 3, 4 and 5, and the ^{1}H-NMR spectra were shown in Appendices 6, 7 and 8.

Compound CM-D3 was light yellow crystal with molecular formula of $C_9H_{10}O_3$, which the molecular weight was 166. The melting point was around 145°C to 147°C. The peak shown in EI MS (m/z) were 166 [M]+, 151 (100), 137, 123. The ^{1}H NMR (300 Hz, $CDCl_3$) (δ_H) data was shown in Table 4.4. After comparing data with those reported in the literature (Table 4.5), it was identified as 2,5-dihydroxy-4-methylacetophenone (Sabui and Venkateswaran, 2003).

Then for compound CM-D4, it was white crystaline with a molecular formula of $C_9H_{10}O_4$, which has a molecular weight of 182. The melting point was around 151°C to 153°C. The peak shown in EI MS (m/z) were 182 [M]+, 167 (100), 152, 139, 124, 111. The ^{1}H NMR (300 Hz, $CDCl_3$) (δ_H) data was shown in Table 4.4. It was identified as 2,5-dihydroxy-4-methyoxyacetophenone after comparing the data with those in the literature as shown in Table 4.6 (Kwon *et al.*, 1999).

For compound CM-D5, it was yellow crystaline with a molecular formula of $C_9H_{10}O_3$, which has a molecular weight of 166. The melting point was around 88°C -89.5°C. The peak data of EI MS (m/z) were 166[M]+, 151(100), 136, 123. And the ^{1}H NMR (300 Hz, $CDCl_3$) (δ_H) data was shown in Table 4.4. It was identified as 3-hydroxy-4-methoxyacetophenone (acetoisovanillone) after comparing the data those in the with literature as shown in Table 4.7 (Kobayashi *et al.*, 1982).

All the above compounds belong to acetophenones. The ^{1}H NMR data of CM-D1 (paeonol) was also shown in Table 4.4 for reference and comparison, which was proved to be an acetophononic compound too. The structures of the acetophenonic compounds purified were showed in Fig. 4.11.



CM-D1 (Paeonol)

CM-D3 2,5-dihydroxy-4 -methylacetophenone

CM-D4 2,5-dihydroxy-4 -methoxyacetopheone

CM-D5 3-hydroxy-4 -methoxyacetophenone (acetoisovanillone)

Fig.4.11 The deduced structures of CM-D1 (paeonol), CM-D3, CM-D4 and CM-D5 according to mass spectra and ^{1}H NMR spectra.

	Chemical Shift (δ ppm)			
Protons	**1**	**2**	**3**	**4**
C2				7.53,s
C3	6.42,s	6.77,s	6.44,s	
C5	6.45, d, *J*=7.5			6.88,d,J=7.2
C6	7.63,d,*J*=7.5	7.10,s	7.20,s	7.54,d,J=7.2
C8	2.56,s	2.56,s	2.53,s	2.54,s
OCH3	3.84,s		3.93,s	3.96,s
CH3		2.27,s		

Table 4.4 Proton assignments of CM-D3, CM-D4 and CM-D5.
s – Singlet; d-Doublet; m – Multiplet; *J* – Coupling constant.

Protons	CM-D3	Literature data (Sabui and Venkateswaran, 2003)
3H, s, C-CH_3	2.27	2.26
3H, s, OCH_3	2.56	2.54
1H, s, H-3	6.77	6.76
1H, s, H-6	7.10	7.09
1H, s, OH	11.86	11.86

Table 4.5 Comparison of proton assignments of CM-D3 with data from the literature.

Protons	CM-D4	Literature data (Kwon *et al.*, 1999)
3H, s, O=C-CH_3	3.93	3.90
3H, s, OCH_3	2.53	2.57
1H, s, H-3	6.44	6.56
1H, s, H-6	7.20	7.25
1H, s, OH	12.49	12.37

Table 4.6 Comparison of proton assignments of CM-D4 with data from the literature.

Protons	CM-D5	Literature data (Kobayashi *et al.*, 1982)
3H, s, O=C-CH_3	3.96	3.91
3H, s, OCH_3	2.54	2.53
1H, s, H-2	7.53	7.50
1H, d, H-5	6.88, J=7.2	6.84
1H, d, H-6	7.54, J=7.2	7.50
1H, s, OH	/	/

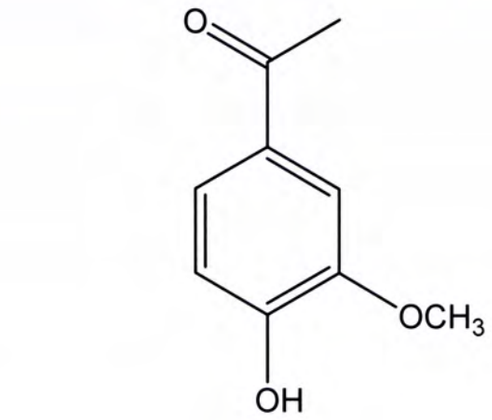
Table 4.7 Comparison of proton assignments of CM-D5 with data from the literature.

4.8 BBMV Glucose Uptake Assay of Acetovallione, CM-D3, CM-D4 and CM-D5

After structure confirmation of CM-C3, CM-D4 and CM-D5, another compound called acetovanillone (4-hydroxy-3-methylacetophenone) (Fig. 4.12), which was also a component found in Cortex Moutan (Li *et al.*, 2004a) with a similar structure with CM-D6, was purchased from Sigma (St. Louis, MO, USA). It was used to compare activities of these fractions and to find out the structure-activity relationship.

4.8.1 Results

From Fig. 4.13, all tested compound had a significant inhibitory effect on BBMV glucose absorption. The structures of CM-D5 and acetovanillone were similar, and they have similar activities in intestinal glucose absorption, which suppressed the glucose absorption by 27% and 25% respectively. For paeonol and CM-D3, they also have similar structures; they showed strong and significantly suppression of BBMV glucose absorption by 64% and 78%, respectively.



4-hydroxy-3-methoxyacetophenone
(acetovanillone)

Fig. 4.12 Structure of acetovanillone.

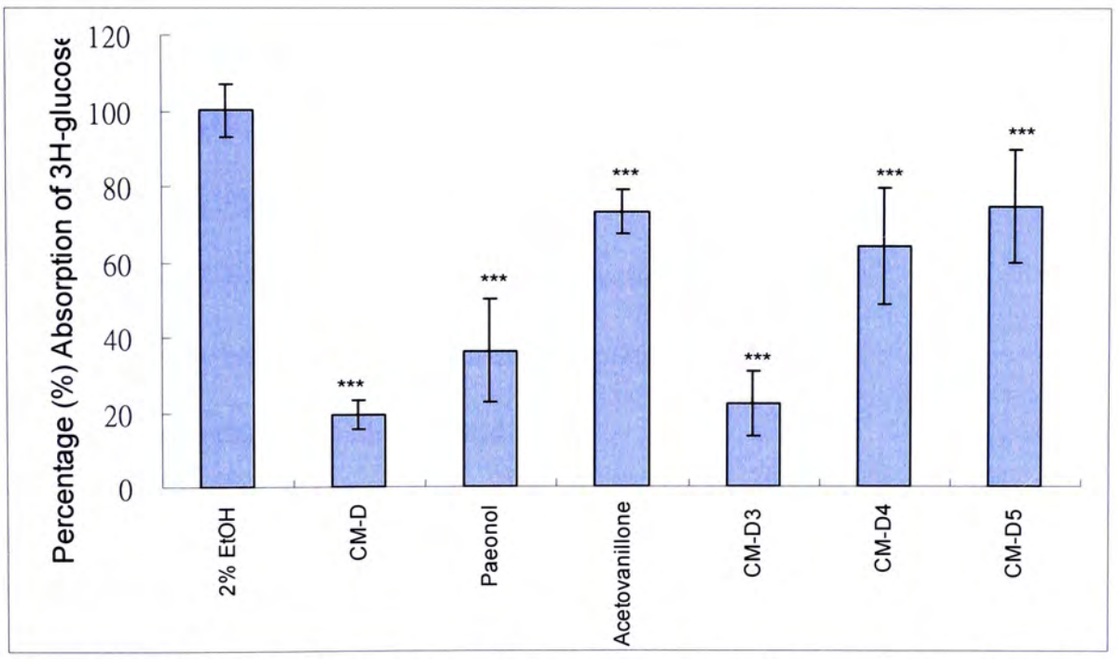


Fig. 4.13 Effects of acetovanillone, CM-D and its sub-fractions on glucose absorption of BBMV. 2% EtOH served as control. The results are expressed as mean±SD, n=12. Asteristo were used to indicate the significantly difference from 2% v/v EtOH: ***$p<0.001$.

4.9 Synthesis of CM-D3s

Although CM-D3 (2,5-dihydro-4-methylacetophenone) was very effective in inhibiting glucose absorption in BBMV, its anti-diabetic effect could not be confirmed by *in vivo* model due to its low extraction yield in CM-D fraction (only with 0.13%). Moreover, it is not available commercially, and so it could not be purchased. Therefore, synthesis of CM-D3 (CM-D3s) was the only way to produce it in relatively large amounts for *in vivo* assays.

4.9.1 Material and Methods

4.9.1.1 Chemicals

Here is a list of the chemicals used in this experiment:

1. Purchased from Aldrich (St. Louis, MO, USA)

   (2,5-diacetoxytoluene) 2-methylhydroquinone diacetate

2. Purchased from Lab-Scan (Bangkok, Thailand):

   Methanol (MeOH), hexane and ethyl acetate (EtOAc).

3. Purchased from BDH Laboratory Supplies (Poole, UK)

   Concentrated hydrochloric acid (HCl)

4.9.1.2 Methods

2-Methyhydroquinone diaacetate, on Fries arrangement followed by monomethylation should produce 2,5-dihydroxy-4-methylacetophenone (Sabui and Venkateswaran, 2003).

A finely powdered mixture of 2-methylhydroquinone diacetate (20g, 96mmol) and anhydrous aluminium chloride (43g, 321 mmol) was heated slowly with stirring in an

oil bath to 110°C -120°C and kept at this temperature for 30 minutes. Then the temperature was increased to 160°C-165°C and kept for 3 hours. The reaction mixture was then cooled and decomposed by 150g of crashed ice, and then with 15ml of concentrated. HCl. The resulting solid was filtered and washed with 30ml of cold distilled water for three times. The product was crystallized from absolute EtOH to yield 2,5-dihydroxy-4-methylacetophenone.

4.9.2 Structure Elucidation of synthesized product

Structure of the synthesized product was elucidated by mass spectrometry (MS) and proton nuclear magnetic resonance (^{1}H-NMR) as described in section 4.8.1, and the result was compared with that of CM-D3.

4.9.3 Results

The amount of product synthesized in the reaction was 1.1864g, and the yield was 5.9%. The product was a pale yellow solid, with its melting point of 147.5°C-148.5°C.

Appendices 9 and 10 showed the results of mass spectrometry and ^{1}H NMR. The peak shown in EI MS (m/z) were 166 [M]+, 151 (100), 137, 123. The ^{1}H NMR (300 Hz, $CDCl_3$) (δ_H) data was shown in Table 4.8. After comparing with data with those reported in the literature, the result matched well with that of CM-D3, and hence it was proved that the product should be 2,5-dihydroxy-4-methylacetophenone (CM-D3).

Protons	Synthesized product	CM-D3	Literature data (Sabui and Venkateswaran, 2003)
3H, s, C-CH_3	2.27	2.27	2.26
3H, s, OCH_3	2.56	2.56	2.54
1H, s, H-3	6.77	6.77	6.76
1H, s, H-6	7.10	7.10	7.09
1H, s, OH	11.87	11.86	11.86

Table 4.8 Comparison of proton assignments of synthesized 2,5-dihydroyl-4-methylacetophenone and CM-D3 with data from the literature.

4.10 Discussion

Sodium paeonol sulfonate was synthesized by reacting with conc. H_2SO_4. It was shown to be water soluble as it was an ionic compound. It had an antibiotic effect (Wang and Tang, 1983; Wu *et al.*, 2003a), inhibition on calcium influx in heart muscle (Tang and Shi, 1991;Wang and Tang, 1983), analgesic, antifebrile and antiphlogistic effects (Wang and Tang, 1983). However, there is no anti-diabetic effect has been reported.

In our study, it did not inhibit glucose absorption in the BBMV assay. The difference between paeonol and sodium paeonol sulfonate was only changing the hydroxyl group on carbon-2 position into sulfonyl group. Therefore, the change in structure and functional group of paeonol would lose the activity in its inhibitory effect on intestinal glucose uptake.

As previous study by another student (Lau, 2004) showed that the inhibitory effect of CM-C on glucose absorption activity was much higher than that of paeonol, hence it was suspected that there should be another active component that exert the inhibitory effect in BBMV assay together with paeonol. Also in Fig. 4.13, it also showed the inhibitory activity of paeonol was not as strong as CM-D fraction thus supporting the previous study.

After extraction with hexane and dichloromethane, it was found that there was another active component found in a range other than that of paeonol that was present in Cortex Moutan organic extracts. The TLC chromatogram showed that the active range should come from a yellow spot (refer to Fig. 4.4, 4.5 and 4.6). We could also see that the amount of that suspected active component was higher in concentration in the dichloromethane extract; thus, isolation of that yellow part was carried out in the dichloromethane extract (CM-D).

All CM-D3, CM-D4, and CM-D5 isolated from CM-D belong to acetophenones, which were the same as paeonol. They showed anti-aggregation activity in rabbit platelets (Lin *et al.*, 1999). They all showed positive and significant results in BBMV assay, and CM-D3 had the greatest extent. This anti-diabetic effect of CM-D3 had been reported before. Very few reports were done on acetophenoic compounds purified from Cortex Moutan as they were only minor components inside Cortex Moutan.

Due to the very low yield in CM-D3 extraction from the herb, synthesis of 2,5-dihydroxy-4-methylacetophenone (CM-D3s) was performed. The product was then used to conduct OGTT to investigate its anti-diabetic effect *in vivo*. Results are shown in the next chapter.

Chapter 5 *In vivo* Studies on Selected Herbs

5.1 Introduction

The herbs showing the most promising effects according to results in the various *in vitro* assays were studied with an *in vivo* model to confirm their anti-diabetic effects. In this project, diabetic animal model was used to conduct the *in vivo* studies in order to investigate the anti-diabetic effect of traditional Chinese herbs. Animal studies were approved by the Animal Experimentation Ethics Committee, CUHK (02/022/MIS).

From the *in vitro* studies, Rhizoma Copitidis, Radix Ophiopogonis, and Cortex Phellodendri were chosen to further study in diabetic animal model. Moreover, Cortex Moutan, Rhizoma Smilacis Chinensis, and Rhizoma Alismatis mixture were also studied in this model to explore their synergistic anti-diabetic effects. And for the compound CM-D3 which purified from Cortex Moutan and acetovallione were also studied in animal model to confirm their *in vitro* results.

5.1.1 Diabetic Animal Models

Animal models have been used extensively in diabetes research for many years, and the research are carried out under control of various legal and ethical restrictions (Rees and Alcolado, 2005). The diabetes animal models available nowadays are including non-human primates, dogs, cats, rats, mice, guinea pigs, rabbits and hamsters (Melby and Altman, 1976; Rees and Alcolado, 2005). Rats and mouse are most commonly used as they are easier to handle and also the cost is lower compared to larger animals. There are two main types of diabetic animal models: chemically induced and genetically defective. For type 1 DM models, it is usually carried out with animals having total insulin deficiency; and for type 2 DM models, it usually has insulin resistance, and

retains the ability to release a certain amount of insulin.

The genetically defective diabetic animal models are made by mutations of several diabetes related genes, such as insulin, insulin receptor, glucokinase and mitochondrial tRNA (Elbein, 1997). Some of the rodent models are monogenic, such as db/db mice (Dong *et al.*, 1997) and zucker fatty rat, and some of them are polygenic, such as Goto-Kakisaki (GK) rat (Sone *et al.*, 2001) and NZO/HlLt mice (Leiter and Reifsnyder, 2004). In recent years, molecular technique produces many new type of knock-in, generalized knock-out and tissue-specific knockout mice, such as IR/IRS-$1^{+/-}$ mice (McIntosh and Pederson, 1999).

For the chemically-induced diabetic models, their pancreatic β-cells are usually partially damaged with diabetogenic toxins such as alloxan, streptozotocin, vacor, dithizone and 8-hydroxyquinolone (Rees and Alcolado, 2005), so that insulin production was suppressed and led to diabetes. In this project, streptozotocin-induced type 2 diabetic rat model was employed. Streptozotocin (STZ, 2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose) is a nitrosurea derivative isolated from *Streptomyces achromogenes* with broad-spectrum antibiotic and anti-neoplastic activity (Bono, 1976). It is commonly used as it has selective destruction ability of pancreatic β-cells (Gunnarsson *et al.*, 1974; Gunnarsson and Hellerstrom, 1973). It is a powerful alkylating agent that has been shown to interfere with glucose transport (Wang and Gleichmann, 1998), glucokinase function (Zahner and Malaisse, 1990). STZ could be injected intraperitoneally (i.p.) and absorbed by the pancreatic β-cells through the glucose transporter 2 (GLUT2) (Elsner *et al.*, 2000), it exerts its effect by several pathways. The main cause of β-cell should be methylation of β-cell DNA, which carbonium ions (CH_3^+) generated by decomposition of the nitrosurea group of STZ (Elsner *et al.*, 2000). STZ also acts as a nitric oxide (NO) donor (Kwon *et al.*, 1994;

Kroncke *et al.*, 1995), that damages β-cell when NO is metabolized (Pieper *et al.*, 1999; Kroncke *et al.*, 1995).

A single large dose of STZ can produce diabetes in rats, probably as a result of direct toxic effects, leading to partially insulinopenic diabetes (Rees and Alcolado, 2005).

5.1.2 Neonatal Streptozotocin-induced Diabetic Rat Model

Type 1 DM would be produced if STZ is injected to adult rats due to total destruction of β-cells (Rodrigues *et al.*, 1999), However, type 2 DM would be produced if STZ is injected to neonatal rats. Regeneration of neonatal β-cells after STZ-induced destruction is incomplete but nevertheless leads to mild to moderate diabetic condition and hyperglycaemia. This β-cells regeneration only happens within a few days after birth, and once the rats grow into adult stage, part of the β-cells activity and its insulin production ability restores at some extent, and has similar situation as type 2 DM (Wang *et al.*, 1996). And when β-cells are destroyed on day 2 (n2) or 5 (n5) instead of the day of birth (n0), β-cells capacity for regeneration declines rapidly. As n0-STZ rats can regenerate more β-cells than n5-STZ rats, the diabetic condition and hence deviation of blood glucose level of n5-STZ rats is more severe than that of the n0-STZ rats (McIntosh and Pederson, 1999). Wistar neonatal-STZ induced diabetic models by intraperitoneal (i.p.) injection were most commonly used (Blondel *et al.*, 1989; Kergoat and Portha, 1985). The pancreatic insulin content of the n5-STZ Wistar rat is 90% depleted at the age of 10 weeks while that of the n0-STZ is only 50% depleted at the same age (Blondel *et al.*, 1989).

The disadvantage of chemically induction is mostly due to the fact that the diabetic status of the rats is not stable enough as time pass by, but the advantages of using

chemical-induced diabetic models are more cost-effective and severe in diabetic condition, hence STZ-induced diabetic rats are used in the project.

5.2 Oral Glucose Tolerance Test (OGTT)

Improving oral glucose tolerance is one of the modes of action for diabetic treatment. For diabetes patients, blood glucose level measured in OGTT should be equal or over 11.0mM. The n0-STZ Wistar rats were used in this study as they have moderate diabetic condition with impair glucose tolerance. Herbal water extract was applied to the rat model and investigated on their oral glucose tolerance improvement effect.

5.2.1 Animal

Neonatal female albino Wistar rats were supplied by and kept in The Laboratory Animal Service Centre, The Chinese University of Hong Kong. Three to four rats were kept in a wire-bottomed cage, in a room with 12-hour light-dark cycle and 22-25°C. Normal rodent diets (Prolab 2500 rodent diet) and tap water were constantly supllied to the rats. Bedding under the cage was changed at least twice a week.

5.2.2 Rat Induction Material and Methods

Rats at day of birth were injected with STZ in citrate buffer (pH 4.5) at a dose of 100 mg/kg body weight in i.p. route. The neonatal rats were grown with their mother until week 6, and then separated from their mother. The rats were ready for use at week 10-12.

Streptozotocin, citric acid monohydrate, citric acid trisodium salt dihydrate, metformin and heparin were purchased from Sigma (St. Louis, MO, USA). Glucose

Assay kits and 100mg/dl (5.56mM) glucose standard were purchased from BioSystems (Barcelona, Spain).

5.2.3 Testing Method for diabetic condition of rats

The 10-12 weeks old (adult stage) n0-STZ diabetic rats were used, and their diabetic condition was confirmed by determining the fasting hyperglycaemic state. The rats with blood glucose over 7.0mM were recruited in the assay. The herbal water extract treatments were given to the rats once daily for eight consecutive days. Oral glucose tolerance test was performed and plasma glucose level of each rat was determined on day 1 for the acute effect study, and also on day 8 for the chronic effect study.

Firstly, rats were randomized into several groups ($n \geq 6$): negative control group (water or 0.6% v/v Tween 80, 5 ml/kg body weight), positive control group (metformin, 200 mg/kg), and different herbal extract treatment groups. The dosages were calculated based on the human equivalent doses.

On day 1 experiment, the rats are fasted for two hours before the experiment started. Then, 300μl of blood samples were collected into a 1.5ml microfuge tube containing heparin (1250U/ml, 5μl/tube) from tail veins of the rats. Once blood samples were taken, the rats were immediately force-fed with different treatments. After 30 minutes, blood samples were taken again and the rats were immediately force-fed with glucose solution (40mg/ml, 2g/kg and 5ml/kg body weight), and the experiment then started and this time was defined as "0 minute". Blood samples were then collected after 15, 30, 45 and 90 minutes. Rats were kept unfed until the last blood sampling was taken. All blood samples should be centrifuged within 30 minutes at 4000×g rpm for five minutes, and the supernatant (plasma) was transferred to another centrifuge tube and stored on ice.

Rats were force-fed with different treatments on day 2 to day 7 without taking blood sample. On day 8, the experimental procedure as same as day 1 was repeated for chronic herb effect studies.

The plasma glucose level was measured by glucose oxidase method. For each reaction, 1ml of glucose assay solution aliquoted inside 1.5ml centrifuge tube was pre-warmed at 37°C. Then, 5µl of sample (plasma sample, distilled water (negative control) and 100mg/dl (5.6mM) glucose standard (positive control)) was added to the warm solution and mixed well. After five miuntes incubation at 37°C, the absorbance at 500nm was measured. The actual glucose concentration in the plasma samples was calculated by calibration with a 5.6mM glucose standard.

Glucose concentration of the plasma sample

= (Absorbance of plasma sample – Absorbance of distilled water) / Absorbance of standard x 100mg/dl

The area under the curve (AUC) of glucose level across time in each day was fitted to multilevel models. Multi-level models were employed to analyze the rate of change of AUC at day 1 and 8 at each time point (t = -30, 0, 15, 30, 45, 60, 90 min) relative to baseline (day 0) between the control group and treatment groups. The public domain software MIXREG (University of Illinois at Chicago, Chicago, IL, USA) was used for fitting multi-level models. All statistical tests were two-sided, with a significant level of 0.05.

5.3.4 Results

The experimental results of OGTT are shown in Fig. 5.1 to 5.6. Metformin (200 mg/kg) which acted as the positive control in the experiment, with plasma glucose level

significantly lower than that of water on both day 1 and day 8, and hence validated the model.

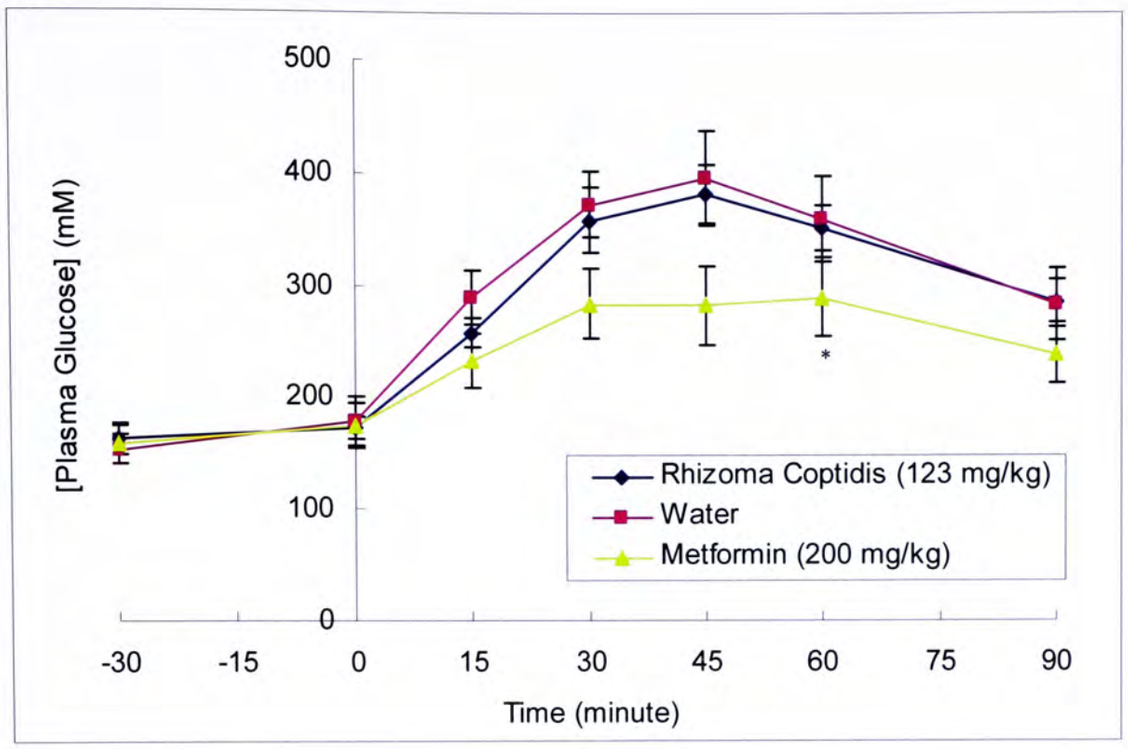
On day 1 of the experiment, no significant difference between water and Rhizoma Coptidis, Cortex Phellodendri, Radix Ophiopogonis and the mixture of Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis in ratio 1:1:1 (400mg/kg) water extract treatment groups was observed as shown in Fig. 5.1, 5.2, 5.3 and 5.4. For Paeonol (200mg/kg), acetovanillone (200 and 400mg/kg) and CM-D3 (400mg/kg) treatment groups as shown in Fig. 5.5, 5.6 and 5.7, they also showed no significant results compared with 0.6% v/v Tween 80 (solvent of the drugs). Our results showed that the above treatment groups did not improve oral glucose tolerance *in vivo* in one day acute treatment. But for paeonol (400mg/kg) treatment group (Fig. 5.5), its plasma glucose level was significantly lower than that of 0.6% v/v Tween 80 at time point 90 min, this showed that paeonol (400mg/kg) treatment could improve oral glucose tolerance *in vivo* after 90 minutes of administration in one day acute treatment.

In day 8 experiment result, no significant difference between water and Rhizoma Coptidis, Cortex Phellodendri, Radix Ophiopogonis and the mixture of Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis in ratio 1:1:1 (400mg/kg) water extract treatment groups was observed as shown in Fig. 5.1, 5.2, 5.3 and 5.4. For acetovanillone (200 and 400mg/kg), as shown in Fig. 5.6, it also showed no significant results compared with 0.6% v/v Tween 80. It is concluded that the above treatment groups did not improve oral glucose tolerance *in vivo* after eight days chronic treatment. For paeonol (200mg/kg) treatment groups as shown in Fig. 5.5, the plasma glucose level was significantly lower than that of 0.6% v/v Tween 80 at time point 30 min; while the plasma glucose level of paeonol (400mg/kg) treatment group was significantly lower than that of 0.6% v/v Tween 80 at time points 15 and 30 min. This showed that paeonol

(200 and 400mg/kg) treatment could improve oral glucose tolerance *in vivo* in both 15 and 30 minutes after an eight day chronic treatment.

After eight days of treatment, body weights of rats in all groups and all sets of experiments increased gradually, and no significant difference was found between treatment groups and the water group (data not shown). No obvious side effect was observed with treatment groups.

(a)



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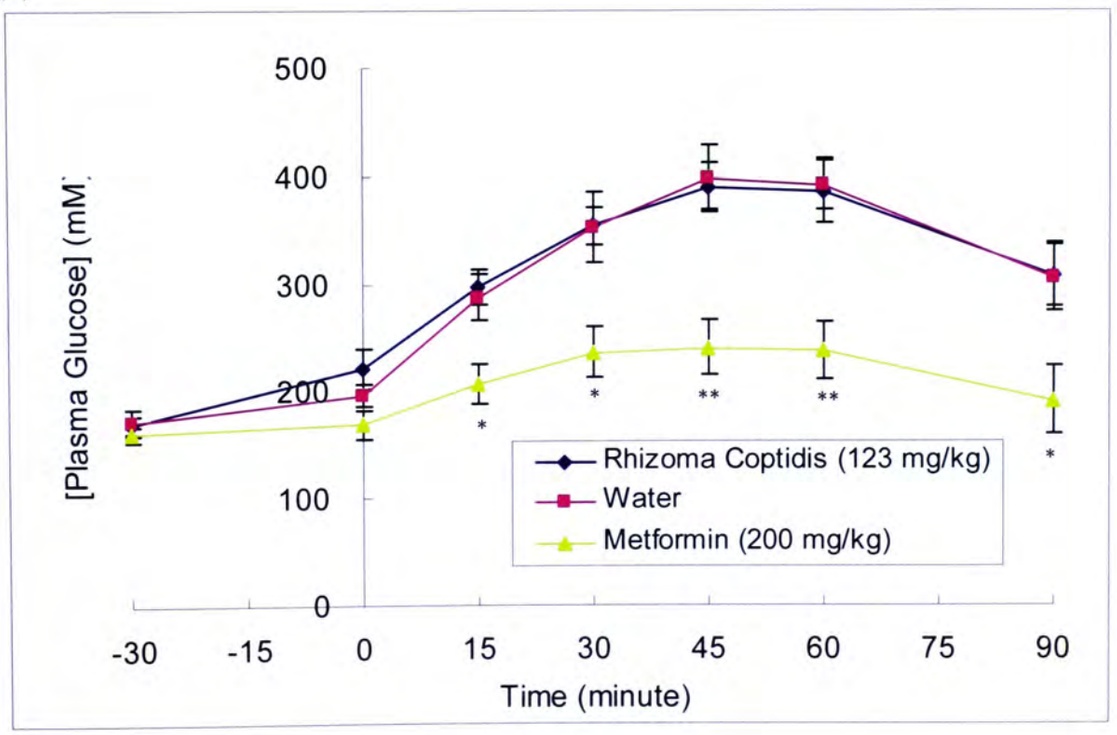
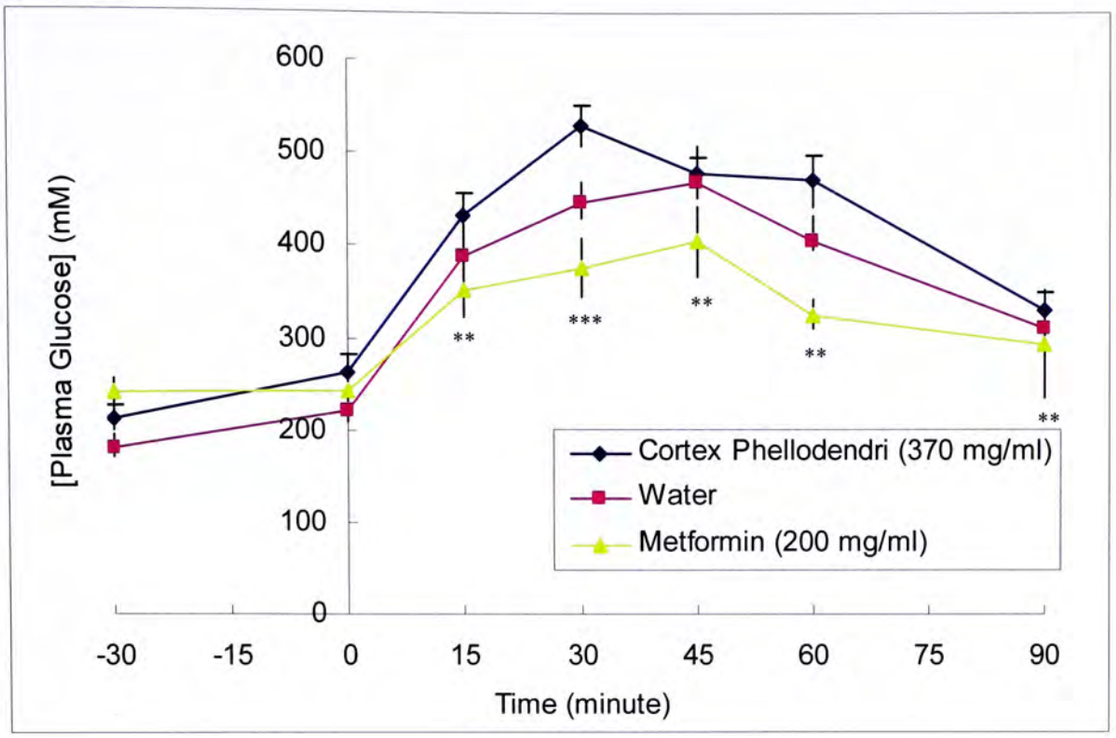


Fig. 5.1 Oral glucose tolerance test of Rhizoma Coptidis (123mg/kg) in Day 1 treatment (upper) and Day 8 treatment (lower). Treatments were administrated to n0-STZ Wistar rats immediately after taking first blood sample (0 min), after 30 min, 40mg/kg glucose solution was force-fed and blood sample was taken at once. Then blood was taken 15, 30, 45, 60 and 90 min from rat tail veins. Metformin (200mg/ml) was performed as the positive control, and water as the negative control. Results were expressed as mean ±SEM, n=7. Asterisles were used to indicate significant difference from water control group: *p<0.05, **p<0.01, ***p<0.001

(a)



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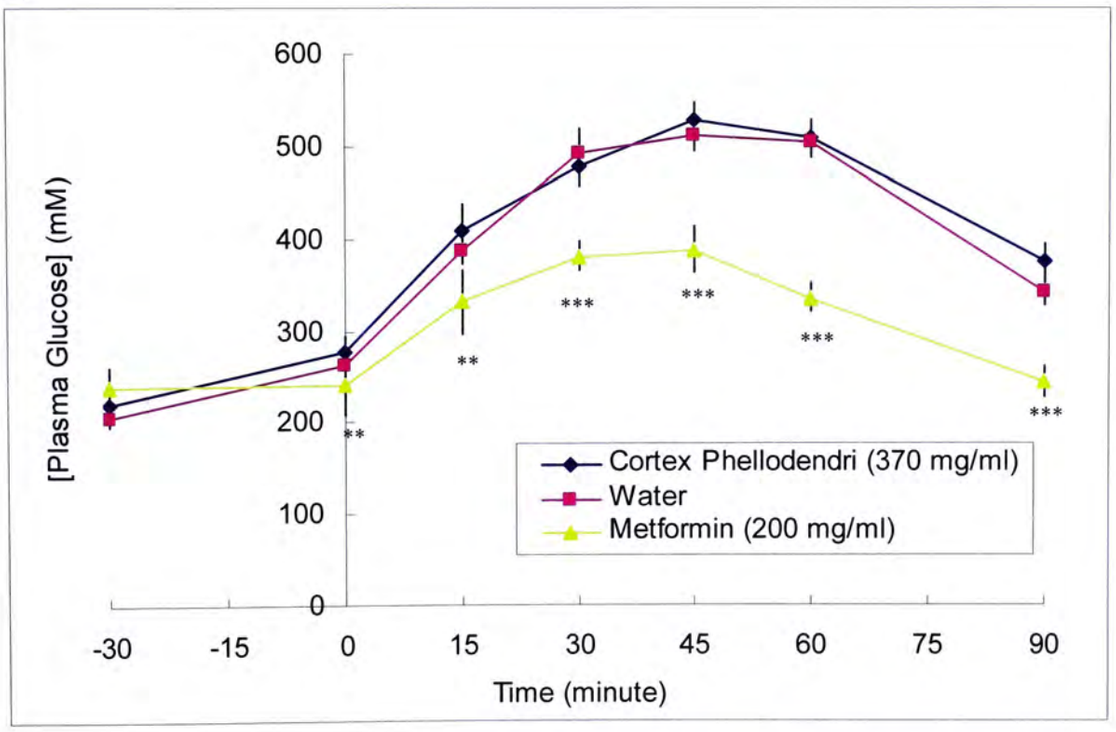
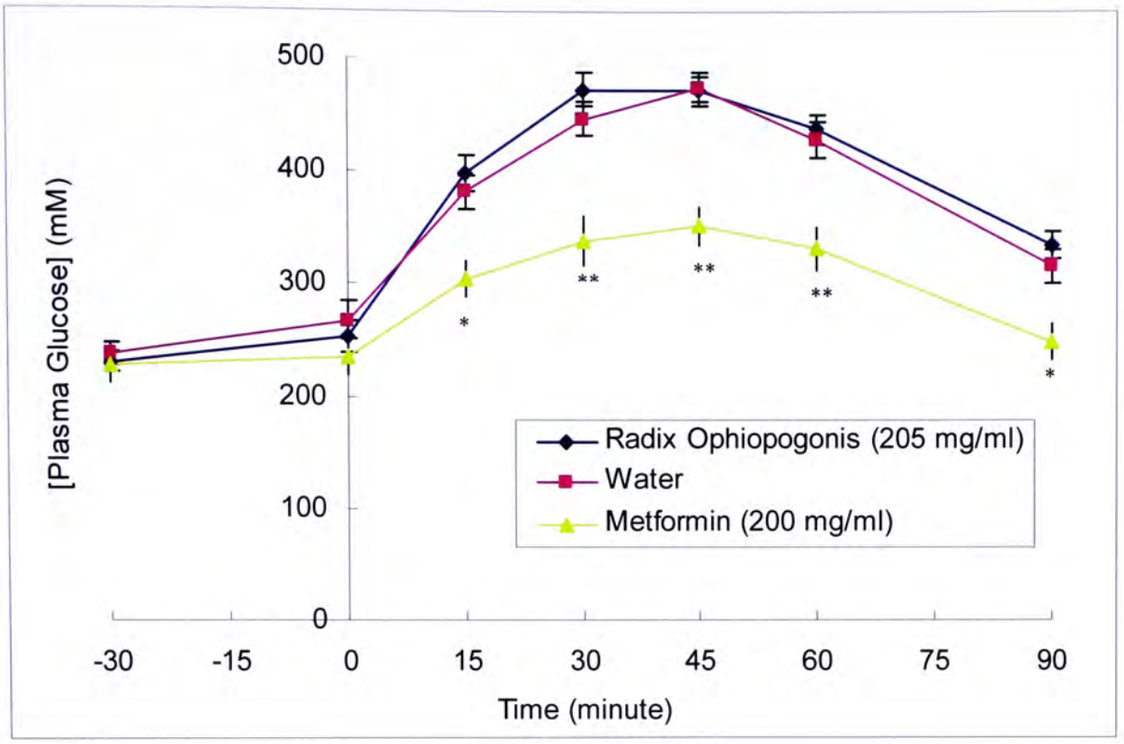


Fig. 5.2 Oral glucose tolerance test of Cortex Phellodendri (370mg/kg) in Day 1 treatment (upper) and Day 8 treatment (lower). Treatments were administrated to n0-STZ Wistar rats immediately after taking first blood sample (0 min), after 30 min, 40mg/kg glucose solution was force-fed and blood sample was taken at once. Then blood was taken 15, 30, 45, 60 and 90 min from rat tail vein. Metformin (200mg/ml) was performed as positive control, and water as negative control. Results were expressed as mean ±SEM, n=11. Asterisles were used to indicate significant different from water control group: *p<0.05, **p<0.01, ***p<0.001

(a)



(b)

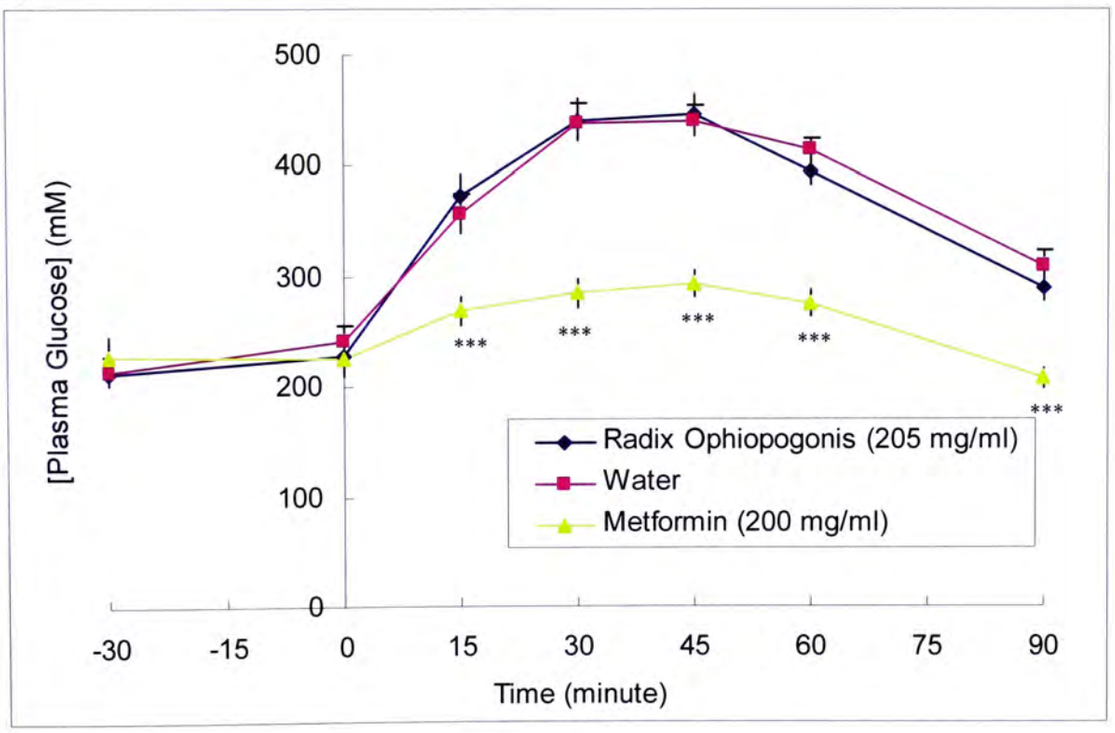
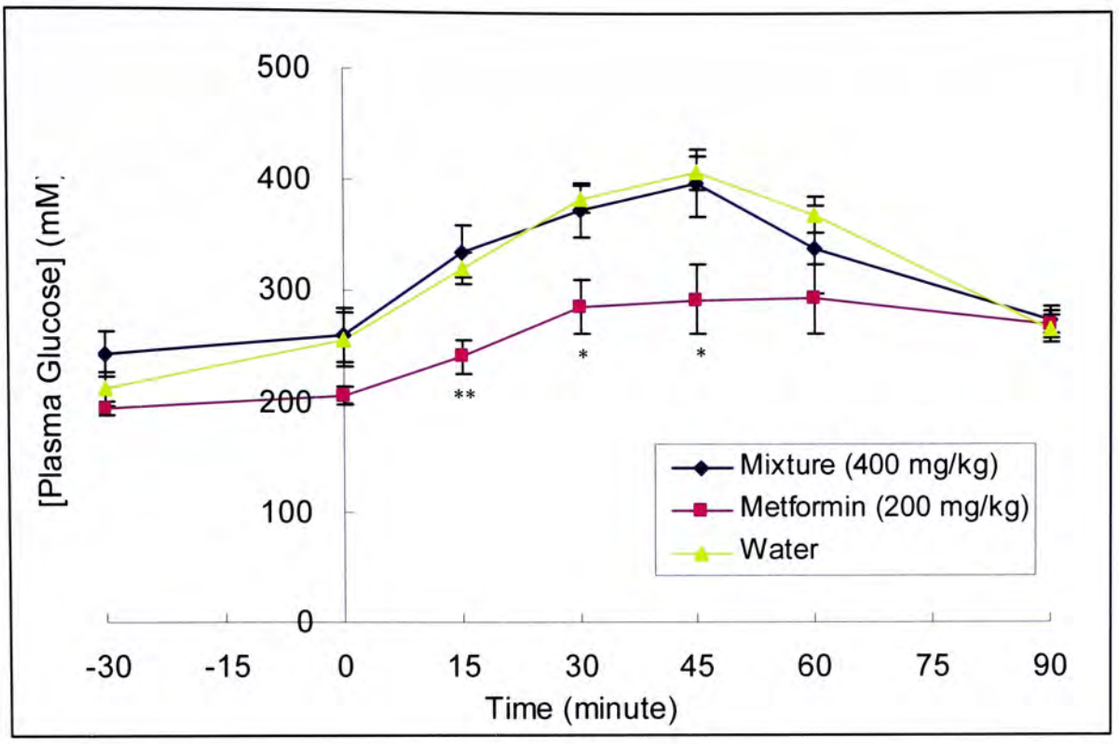


Fig. 5.3 Oral glucose tolerance test of Radix Ophiopogonis (205mg/kg) in Day 1 treatment (upper) and Day 8 treatment (lower). Treatments were administrated to n0-STZ Wistar rats immediately after taking first blood sample (0 min), after 30 min, 40mg/kg glucose solution was force-fed and blood sample was taken at once. Then blood was taken 15, 30, 45, 60 and 90 min from rat tail vein. Metformin (200mg/ml) was performed as positive control, and water as negative control. Results were expressed as mean ±SEM, n=12. Asterisles were used to indicate significant different from water control group: *p<0.05, **p<0.01, ***p<0.001

(a)



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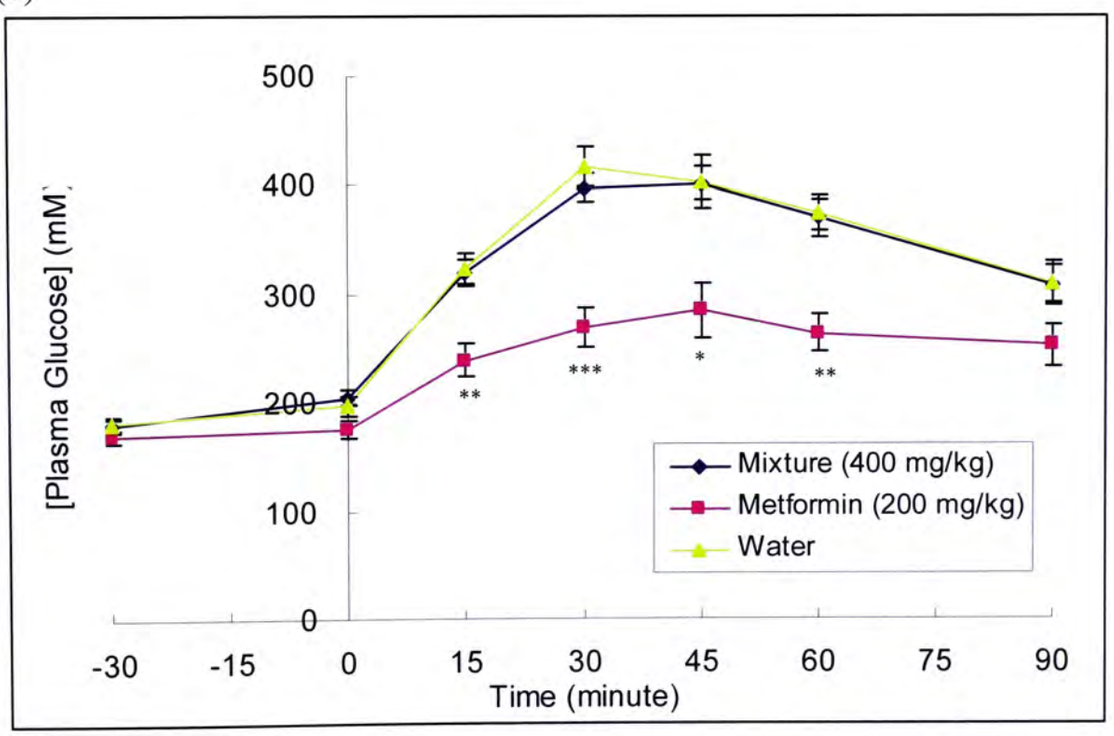

Fig. 5.4 Oral glucose tolerance test of mixture of Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis in ratio 1:1:1 (400mg/kg) in Day 1 treatment (upper) and Day 8 treatment (lower). Treatments were administrated to n0-STZ Wistar rats immediately after taking first blood sample (0 min), after 30 min, 40mg/kg glucose solution was force-fed and blood sample was taken at once. Then blood was taken 15, 30, 45, 60 and 90 min from rat tail vein. Metformin (200mg/ml) was performed as positive control, and water as negative control. Results were expressed as mean ±SEM, n=12. Asterisles were used to indicate significant different from water control group: *p<0.05, **p<0.01, ***p<0.001

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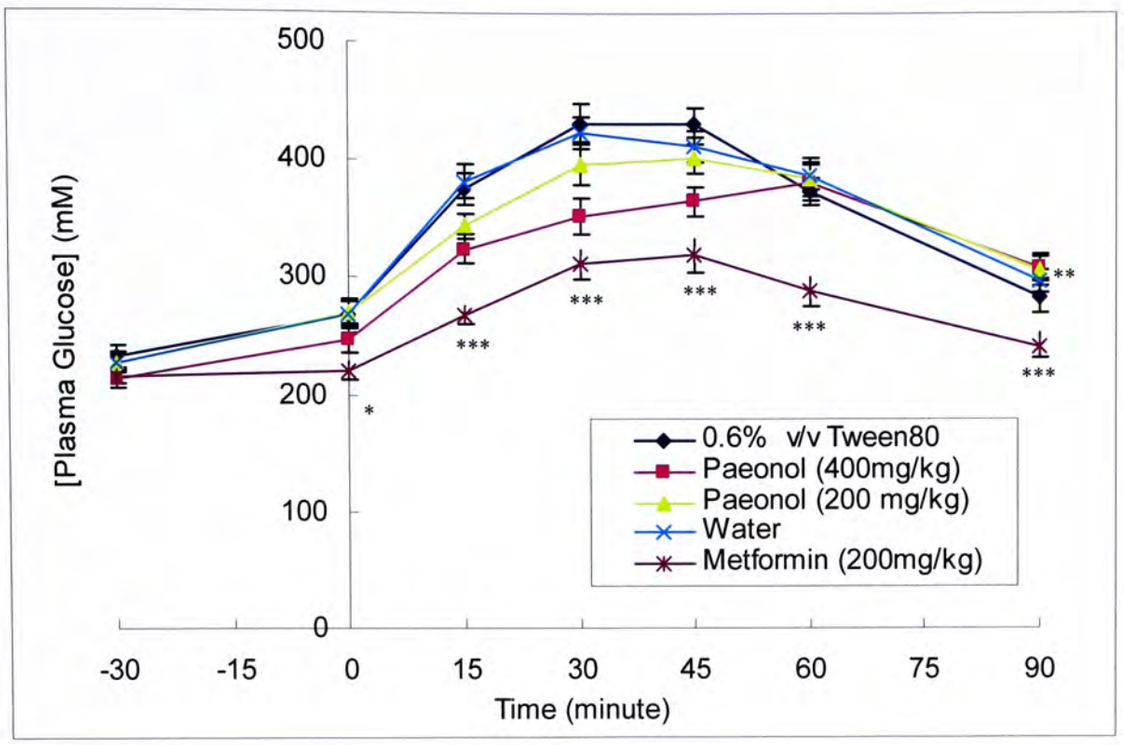

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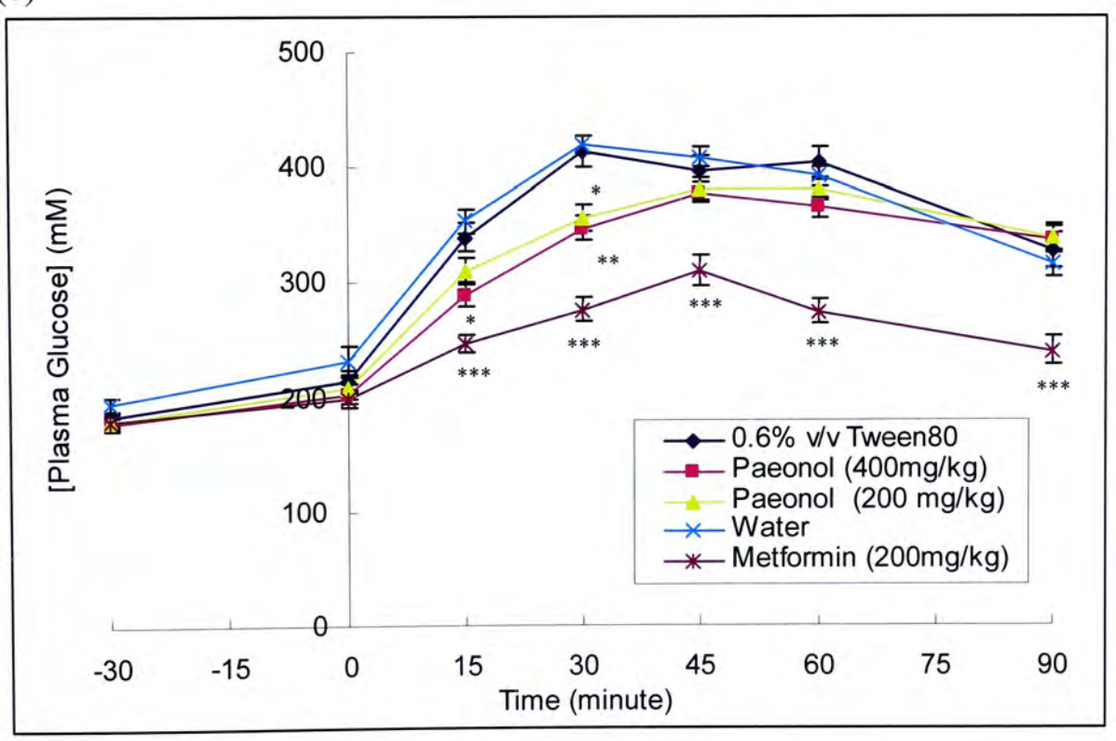
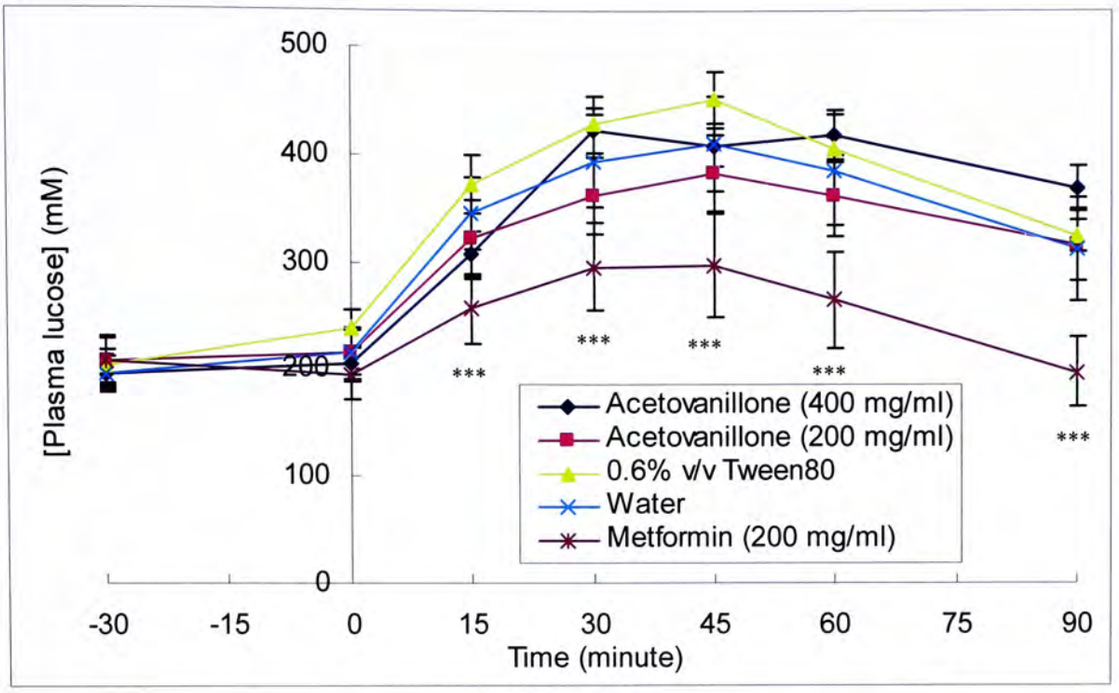


Fig. 5.5 Oral glucose tolerance test of Paeonol (200 and 400mg/kg) in Day 1 treatment (upper) and Day 8 treatment (lower). Treatments were administrated to n0-STZ Wistar rats immediately after taking first blood sample (0 min), after 30 min, 40mg/kg glucose solution was force-fed and blood sample was taken at once. Then blood was taken 15, 30, 45, 60 and 90 min from rat tail vein. Metformin (200mg/ml) was performed as positive control, and water as negative control. Results were expressed as mean ±SEM, n=12. Asterisles were used to indicate significant different from water control group: *p<0.05, **p<0.01, ***p<0.001

(a)



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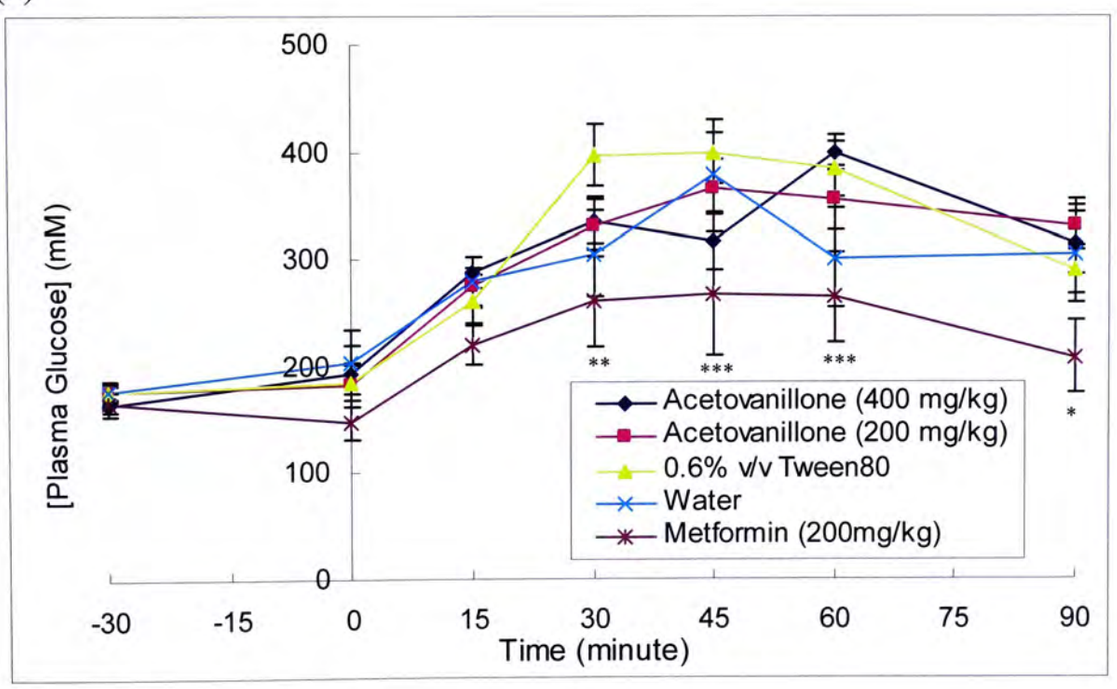


Fig. 5.6 Oral glucose tolerance test of Acetovallione (200 and 400mg/kg) in Day 1 treatment (upper) and Day 8 treatment (lower). Treatments were administrated to n0-STZ Wistar rats immediately after taking first blood sample (0 min), after 30 min, 40mg/kg glucose solution was force-fed and blood sample was taken at once. Then blood was taken 15, 30, 45, 60 and 90 min from rat tail vein. Metformin (200mg/ml) was performed as positive control, and water as negative control. Results were expressed as mean ±SEM, n=7. Asterisles were used to indicate significant different from water control group: *p<0.05, **p<0.01, ***p<0.001

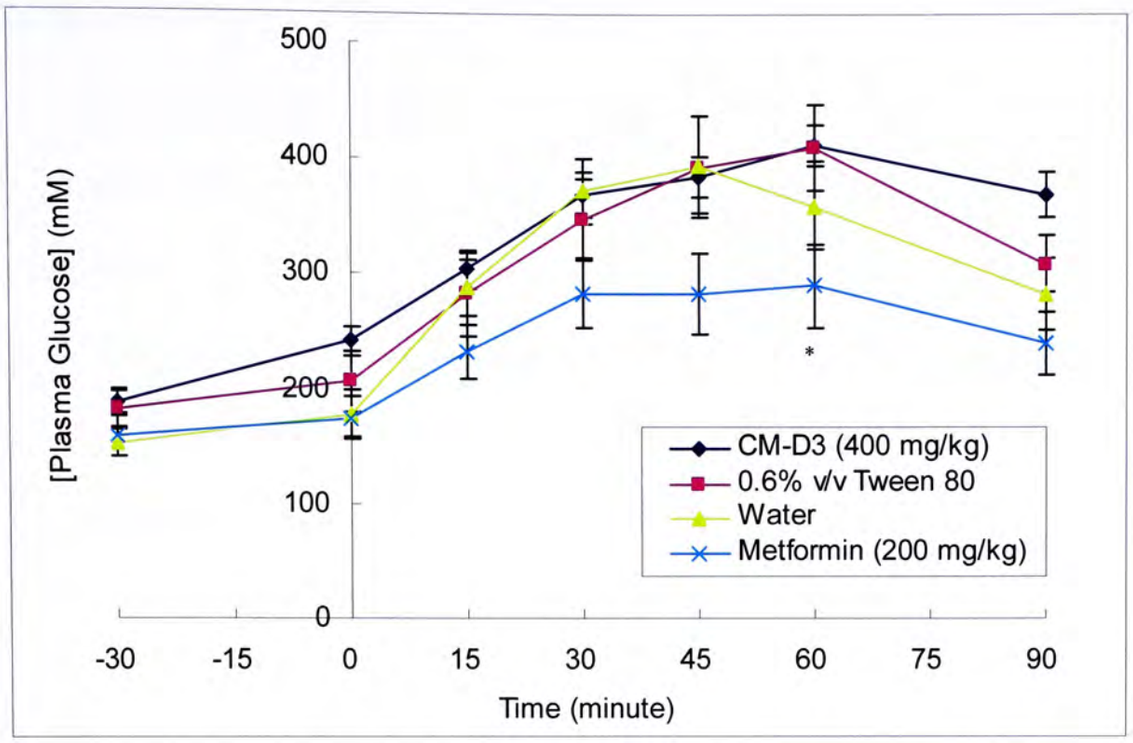


Fig. 5.7 Oral glucose tolerance test of CH-D3 (400mg/kg) in Day 1 treatment. Treatments were administrated to n0-STZ Wistar rats immediately after taking first blood sample (0 min), after 30 min, 40mg/kg glucose solution was force-fed and blood sample was taken at once. Then blood was taken 15, 30, 45, 60 and 90 min from rat tail vein. Metformin (200 mg/ml) was performed as positive control, and water as negative control. Results were expressed as mean ±SEM, n=8. Asterisles were used to indicate significant different from water control group: *p<0.05, **p<0.01, ***p<0.001

5.3 Basal Glycaemia Test

Lowering the basal glycaemic blood glucose level could be a method in improving the diabetic status of the patients. Basal glycaemia test was performed to study the effect of herbal extracts on the basal fasting plasma glucose. In this study, n5-STZ Wistar rats were used instead of n0-STZ Wistar rats as they had higher basal glycaemia.

5.3.1 Animal

Neonatal female albino Wistar rats were used and the method of maintenance was the same as described in section 5.2.1.

5.3.2 Rat Induction Material and Methods

Diabetes was induced in rats at the fifth day after birth. Rats were injected with STZ in citrate buffer (pH 4.5) at a dose of 70 mg/kg body weight in i.p. route. The neonatal rats grew with their mother until week 6, and they were then separated from their mother. The rats were ready for use at week 10-12.

5.3.3 Testing Method

The 10-12 weeks old (adult stage) n5-STZ diabetic rats were used, and their diabetic condition was confirmed by determining the fasting hyperglycaemic state. The rats with blood glucose over 7.0mM were recruited in the assay. The herbal water extract treatments were given to the rats once daily for eight consecutive days. Oral glucose tolerance test was performed and plasma glucose level of each rat was determined on day 1 for the acute effect study, and it was also done on day 8 for the chronic effect study.

Firstly, rats were randomized into several groups ($n \geq 6$): negative control group

(water, 5ml/kg body weight), positive control group (metformin, 200mg/kg), and herbal water extract treatment groups (123mg/kg Rhizoma Coptidis, 307mg/kg Cortex Phellodendri and 205 mg/kg Radix Ophiopogonis). The dosages were calculated based on the human equivalent doses.

On day 1 of the experiment, the rats are fasted for two hours before the experiment started. Then, 300μl of blood samples were collected into a 1.5ml microfuge tube containing heparin (1250U/ml, 5μl/tube) from the tail veins of the rats. Once blood samples were taken, the rats were immediately force-fed with different treatments, and the experiment started and this time was defined as "0 minute". Blood samples were then collected after 45, 90, 135, 180, 240 and 360 minutes. Rats were kept unfed until the last blood sampling was taken. All blood samples should be centrifuged within 30 minutes at 4000×g rpm for five minutes, and the supernatant (plasma) was transferred to another centrifuge tube and stored on ice.

Rats were force-fed with different treatments on day 2 to day 7 without taking blood sample. On day 8, the experimental procedure as the same as day 1 and was repeated for the chronic herb effect study.

The plasma glucose level was measured by using the glucose oxidase method. For each reaction, 1ml of glucose assay solution aliquoted inside 1.5ml centrifuge tube was pre-warmed at 37°C. Then, 5μl of sample (plasma sample, distilled water (negative control) and 100mg/dl (5.6mM) glucose standard (positive control)) was added to the warm solution and mixed well. After five miuntes incubation at 37°C, the absorbance at 500nm was measured. The actual glucose concentration in plasma sample was calculated by calibration with a 5.6mM glucose standard.

Glucose concentration of the plasma sample

= (Absorbance of plasma sample – Absorbance of distilled water) / Absorbance of standard x 100mg/dl

The area under the curve (AUC) of glucose level across time in each day was fitted to multilevel models. Multi-level models were employed to analyze the rate of change of AUC at day 1 and 8 at each time point (t = 45, 90, 135, 180, 240, 360 min) relative to baseline (day 0) and the results were compared between the control group and treatment groups. The public domain software MIXREG (University of Illinois at Chicago, Chicago, IL, USA) was used for fitting multi-level models. All statistical tests were two-sided, with a significant level of 0.05.

5.3.4 Results

The experimental results of basal glycaemia test are shown in Fig. 5.8. Metformin (200 mg/kg), which acted as positive control in the experiment, the plasma glucose level was significantly lower than that of water on both day 1 and day 8, and hence validated the model.

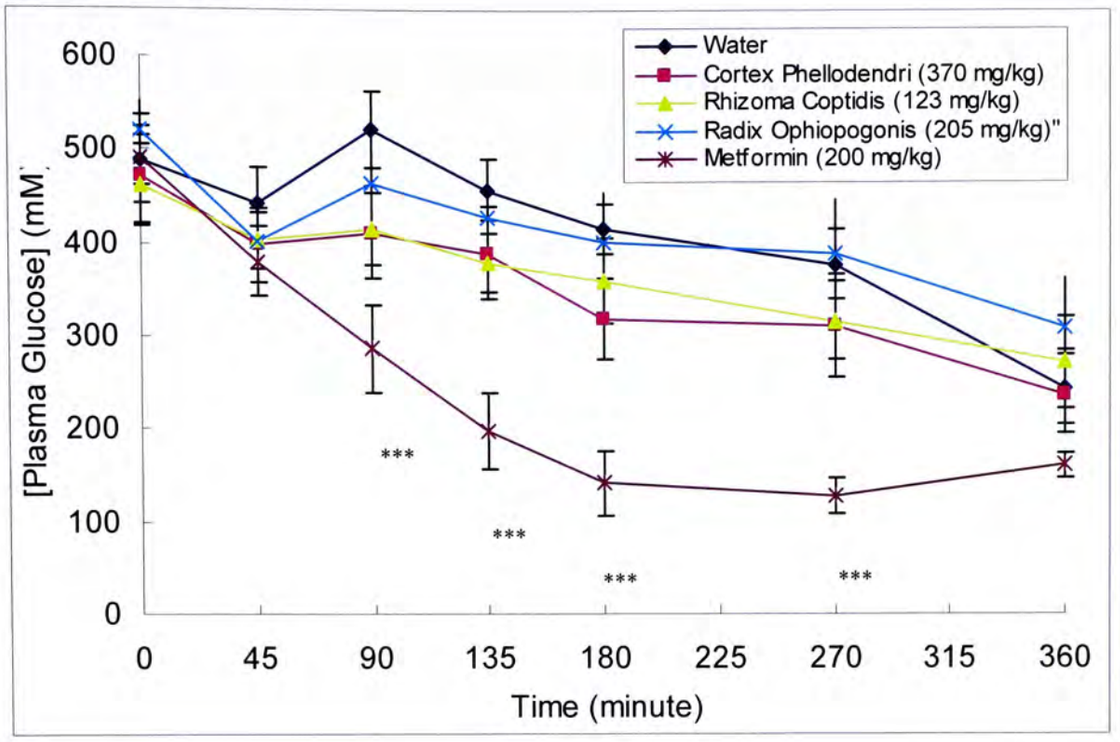
On day 1 of the experiment, no significant difference between water and three herbal treatment groups were observed (Rhizoma Corptidis, Cortex Phellodendri and Radix Ophiopogonis). It showed that three of the above herbal water extracts did not exert any hypoglycaemic effect on the basal glycaemia *in vivo*.

On day 8 of the experimental, the plasma glucose level of Rhizoma Corptidis treatment group was significantly higher than that of the water group at time point t=240 and 360 min; also the plasma glucose level of Cortex Phellodendri treatment group was significantly higher than that of the water group at time point t=360. On the other hand, the plasma glucose level of Radix Ophiopogonis treatment group did not

show significant difference with that of water group. Hence it concluded that the herbal water extracts did not exert any hypoglycaemic effect on the basal glycaemia *in vivo*.

After eight days of treatment, body weights of rats in all groups increased gradually, and no significant difference was found between treatment groups and the water group (data not shown). No obvious side effect was observed with the treatment groups.

(a) Day 1 Experiment



(b) Day 8 Experiment

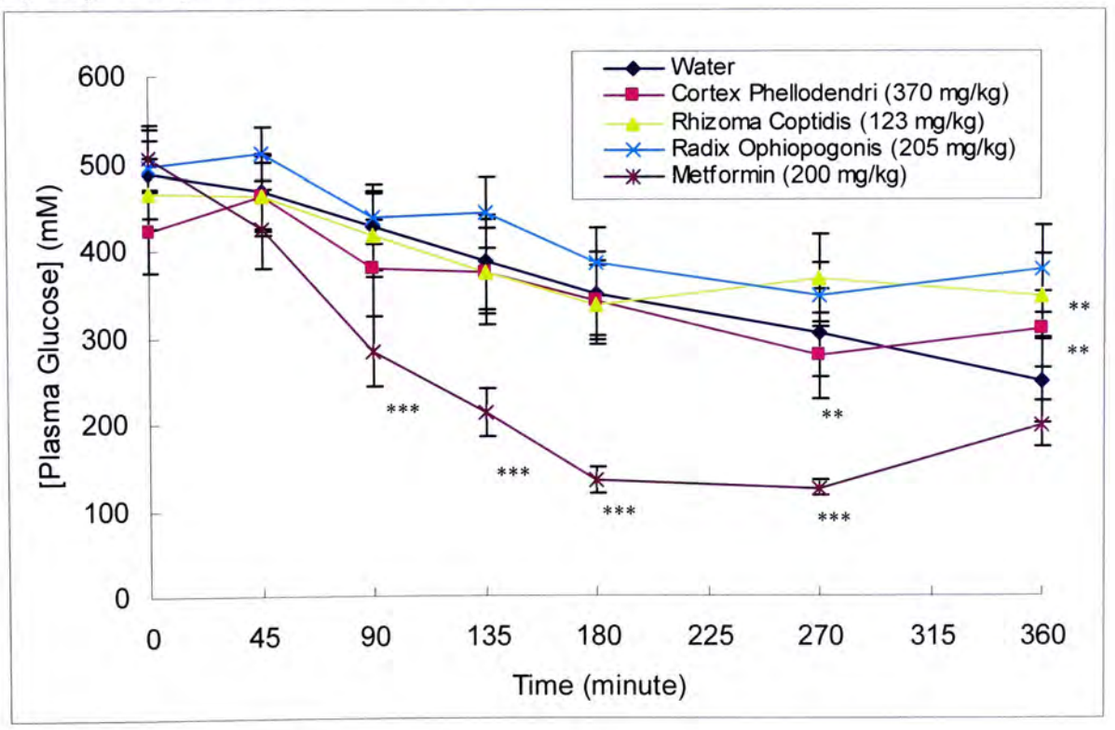


Fig. 5.8 Basal glycaemia test with Cortex Phellodendri, Rhizoma Coptidis, Radix Ophiopogonis in Day 1 experiment (upper) and Day 8 experiemnt (lower). Treatments were given to n5-STZ Wistar rats immediately after taking first blood sample (0 min), then blood was taken 45, 90, 135, 180, 270 and 360 min after treatment administrated. Metformin (200mg/ml) was performed as positive control, and water as negative control. Results were expressed as mean ±SEM, n=8. Asterisles were used to indicate significant different from water control group: *p<0.05, **p<0.01, ***p<0.001

5.4 Discussion

The neonatal STZ Wistar rat models were well recognized type 2 diabetes animal model (Giroix *et al.*, 1983; Schaffer and Mozaffari, 1999). The glucose induced insulin secretion is defective in STZ model by destroying pancreatic β-cells (Rodrigues *et al.*, 1999). In this project, the n0-STZ rats were used in the oral glucose tolerance test while the n5-STZ rats were used in the basal glycaemia test. The n5-STZ rats have more severe hyperglycaemia and higher basal glycaemia level then n0-STZ, and hence provide a sensitive model for investigating the herbal effect on hypoglycemic study. On the other hand, n0-STZ rats have moderate hyperglycaemia and impaired oral glucose tolerance which has a similar glycaemic level comprared to human type 2 diabetes, hence suitable for oral glucose tolerance test. Both models are glucose intolerance, however, insulin resistance is only observed in n5-STZ (Blondel *et al.*, 1990).

Metformin is an oral hypoglycemic agent, which belongs to the class known as the biguanides. Metformin reduces fasting plasma glucose concentration by reducing rate of hepatic glucose production via gluconeogenesis and glycogenolysis. (Ashokkumar and Pari, 2005). In this animal study, metformin significantly lowered the basal glycaemia and improved oral glucose tolerance in neonatal-STZ rats in all set of experiment, hence validated the model. Also, several other oral anti-diabetic agents with different modes of action, including sulfonylureas and α-glucosidase inhibitors also can validate this STZ rat model (Portha and Serradas, 1991; Degirmenci *et al.*, 2002), therefore, this is a good animal model which is suitable for studying the anti-diabetic effect of potential drug candidates.

Rhizoma Coptidis, Cortex Phellodendri and Radix Ophiopogonis were chosen from the previous *in vitro* studies as they have shown the most potent anti-diabetic effects in those *in vitro* studies, and their effects need to be further confirmed by an *in vivo* model.

Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis were studied in this assay as they have proven to have promising anti-diabetic effects on various *in vitro* studies which were done by other students (Chan, 2004; Lau, 2004). Each individual herbal water extract was already proved to have no improving oral glucose tolerance ability by other students. Then in this project, the same amount of each herb was mixed together and their synergistic effects were studied. For acetovanillone and CM-D3, they are the active components in Cortex Moutan, and acetovanillone did not show any anti-diabetic effect in the BBMV assays while CM-D3 did. They were subjected to this *in vivo* assay to confirm the *in vitro* results.

Among the tested treatments, the water extracts of Rhizoma Coptidis (123mg/kg), Cortex Phellodendri (370mg/kg), Radix Ophiopogonis (205mg/kg), mixture of Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis in ratio 1:1:1 (400mg/kg), and also pure compounds inside Cortex Moutan acetovanillone (200 and 400mg/kg) and CM-D3 (400mg/kg), all did not show any positive effects in OGTT; that is, they have no anti-hyperglycaemic effect. The mixture of Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis in ratio 1:1:1 (400mg/kg) did not give any synergistic effect on improving oral glucose tolerance.

Dur to low extraction yield of CM-D3 from Cortex Moutan raw herb, synthere of CM-D3 was done. Since the amount of product CM-D3s was not enough for chronic study in rat model, only 1 day acute treatment could be studied.

Furthermore, the water extracts of Rhizoma Coptidis (123mg/kg), Cortex Phellodendri (370mg/kg), and Radix Ophiopogonis (205mg/kg) did not give any positive effects in basal glycaemia test. But for paeonol (200 and 400mg/kg), it showed positive effects on OGTT by both acute (1 day) and chronic (8 days) treatment. Paeonol (400mg/kg) had better effect on improving oral glucose tolerance than paeonol

(200mg/kg) in its chronic treatment.

The dosage used in herbal water extracts was equivalent to the human dosage recorded in *Zhong Hua Ben Cao* (中華本草) and the State Pharmacopoeia Commission (State Pharmacopoeia Commission, 2000). Since the dosage reported is for raw herb, hence the amount of herbal treatments in the tests of this project are already very high as herb extract were used.

Comparing with the various results of *in vitro* studies, all the above treatments did not exhibit any acute or chronic anti-diabetic effects *in vivo*. The great difference between the *in vitro* results and *in vivo* results might be due to several possible reasons, including the presence of glucose in the herbs which will hinder their anti-diabetic effects; active component(s) inside herbs could not be absorbed through the digestive system; the active component(s) is broken down during digestion; the dosage of each treatment was not suitable. The dosage was, although equivalent to human dosage, still not correlated to the results of *in vitro* studies; also it is difficult to calculate the dosage used from *in vitro* tests and then apply to the *in vivo* model. Variations between individual rats would also decrease the power of statistical analysis, and large sample size should be employed in order to achieve the reliable results with lower standard deviation. Due to the presence of a small amount of glucose inside the herb, the active component(s) could not show strong anti-diabteic effect in the animal model. Metformin, as a pure compound, on the other hand could show a significant anti-diabetic effect in this animal model.

The limitations of this STZ diabetic rat model could also take part in these disappointing results. Hypoinsulinaemia is present instead of hyperinsulinaemia in this model as STZ chemically destroy pancreatic β-cells. Insulin resistance was only found in n5-STZ rats but not in n0-STZ rats, so n0-STZ rats cannot totally represent the real

situation in type 2 diabetes patients. As mentioned before, the blood glucose level, i.e. diabetic status of the STZ diabetic rats are not stable, the blood glucose will increase as the age of rats increased (over 14 weeks).

To improve the results and for further study, genetic type 2 diabetic animal models, such as *db*/*db* mice, Zucker fatty rats and Goto-Kakisaki rats could be used.

Chapter 6 General Discussion

6.1 Introduction

Type 2 DM is a major health problem worldwide with great prevalence (King *et al.*, 1998). Many western oral medications were invented to control blood glucose level by means of different mode of actions, but they have adverse side effects. New medicine is needed with less side effects and better effectiveness. Traditional Chinese herbs are used for many years and with much less side effects.

A common complication among elderly patients suffering from diabetes mellitus is ulceration of the foot. Considering the disadvantages of limb amputation of DM patients who have serious foot ulcer with uncontrolled blood glucose level, two Chinese herbal formulae were designed by an herbalist from the Shanghai Institute of Integrated Western and Chinese Medicine, in order to reduce the chance of limb amputation by improving the wound healing. Formula 1 (托毒生肌湯) was designed to strengthen muscle and control swelling; Formula 2 (薯味地黃湯) was used to promote regeneration of muscle and tissues (Wong *et al.*, 2001). The herbs were listed in table 1.3. But the original reason of foot ulcer is diabetes mellitus, hence investigate of those herbs on their anti-diabetic effects could be another way to reduce the chance of foot ulcer by lower the blood glucose level of those patients.

There are also another traditional herbal formulae used in China for many years in treating DM. These twelve herbal formulae are listed in appendix 1. Fourteen herbs were chosen from these formulae according to their dosage and frequency of use among twelve formulae (Table 1.2).

By using scientific approach, investigation of anti-diabetic effect of different

traditional Chinese herbs was done.

1. Screening the potential anti-diabetic herbs with the use of four *in vitro* models, namely H4IIE hepatoma cells, BBMV, Hs68 human foreskin fibroblasts and 3T3-L1 adipocytes. They represented different mode of actions in regulating blood glucose level, including inhibition of gluconeogenesis, suppression of intestinal glucose absorption and enhancement glucose uptake into peripheral tissues.
2. The most potent anti-diabetic herb was further purified to find out its active component(s).
3. *In vivo* neonatal STZ diabetic Wistar rat model was used to confirm the *in vitro* results.

In vitro studies of Formula 1 and Formula 2 were completed by other two colleagues (Chan, 2004;Lau, 2004). And the results were showed in Table 6.1.
From the results, Rhizoma Smilacis Chinensis from Formula 1, Cortex Moutan and Rhizoma Alismatis from Formula 2 had most potent anti-diabetic effect *in vitro*. They were chosen out, and further studied in animal model and/or investigated the active compound(s) inside the herb which gave the effect.

In this project, other fourteen selected herbs from traditional herbal formulae were studied using same *in vitro* techniques and screening approach. Three herbs with most potent effect were Cortex Phellodendri, Rhizoma Copitidis and Radix Ophiopogonis from *in vitro* assays.

The criteria of choosing the effective herbs based on:

1) Positive results in all *in vitro* studies:

The reasons of using four *in vitro* assays as screening mechanism because these four models could mimic the glucose homeostasis mechanism inside human body; also

Chinese medicine is focus on the improvement of whole body rather than one target organ or mechanism. Hence if the herb could give all positive results among four models, which mean the chosen herb is all-rounded and worth further studying in animal model.

2) Low effective dosage:

   The herb with lower effective dosage was preferred rather than with high effective dosage. It could prevent the toxicity of the herbal extract, and also be more cost effective.

3) Toxicity of the herb

   The herb should have less or no toxicity according to pharmacopoeia.

According to these criteria, six herbs were chosen out (three from Formula 1 and 2, three from other formulae) (Table 6.2). Due to limiting time, only three herbs could be chosen from each part. It was reasonable as the final aim of this project is to find out the possibility to make a new herbal formula in treating DM, and the new formula should be with less than five herbs.

Herb Name	H4IIE	BBMV (Hexane)	Hs68	3T3-L1
Formula 1 (Chan, 2004)				
Radix Astragali	- 9.5% (0.04)	- 35.4% (1.0) *	+ 40.1% (1.0) *	+ 94.6% (0.1) *
Radix Rehmanniae	+ 32.9% (1.0)	-2.05% (1.0)	+ 33.0% (10.0) *	+ 78.3% (0.01) *
Rhizoma Smilacis Chinensis	- 81.9% (5.0) *	-7.36% (1.0)	+ 51.8% (0.01) *	+ 87.7% (0.1) *
Rhizoma Atractylodis Macrocephalae	- 26.2% (1.0)	- 66.3% (1.0) *	+ 26.6% (0.01) *	+ 43.3% (0.1) *
Radix Polygoni Multiflori Preparata	- 76.7% (5.0) *	-1.12% (1.0)	+ 43.3% (1.0) *	+ 55.4% (0.01) *
Radix Stephaniae Tetrandrae	- 5.7% (0.2)	- 44.1% (1.0) *	- 54.2% (0.001) *	+ 11.0% (0.1)
Formula 2 (Lau, 2004)				
Cortex Moutan	-73.6% (5.0)	-80.0% (1.0) ***	+24.3% (0.01) *	+78.9% (0.01) ***
Fructus Corni	-17.5% (5.0)	-2.2% (1.0)	+32.8% (0.01) ***	+44.3% (0.1) *
Fructus Schisandrae Chinensis	+258.1% (5.0)	-30.8% (1.0) ***	+44.9% (1.0) ***	+47.4% (0.01) ***
Poria	+116.8% (5.0)	-33.7% (1.0) ***	+9.9% (1.0)	+47.4% (1.0) ***
Radix Astragali	+88.5% (1.0)	-33.6% (1.0) ***	+40.1% (1.0) ***	+94.6% (0.1) ***
Radix Rehmanniae	+150.2% (0.2)	-0.7% (1.0)	+33.0% (10.0) ***	+78.3% (0.01) ***
Rhizoma Alismatis	-22.6% (5.0)	-8.2% (1.0) **	+109.3% (0.1) ***	+82.8 (1.0) ***
Rhizoma Dioscoreae	+74.6% (5.0)	-16.5% (1.0) ***	+93.2% (10.0) ***	+10.6% (0.01)

Table 6.1 Summary of *in vitro* studies of fourteen traditional Chinese herbs from Formula 1 and Formula 2. The number blanketed represents the concentration in mg/ml aqueous extract, except in BBMV studies with $CHCl_3$ extract of aqueous extract. Significantly different from control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.2 Summary of Research Findings

Before any experimental studies were carried out, authentication was performed to prove that all the selected herbs were genuine.

Among 14 herbs tested, three herbs were selected out namely Cortex Phellodendri, Rhizoma Corptidis and Radix Ophiopogonis. They posses the most potent anti-diabetic effect among different *in vitro* screening systems (table 3.1).

Sodium paeonol sulfonate was synthesized in order to improve the solubility of paeonol in water. However, it did not exert the same results as paeonol did in BBMV assay. Then purification of active components in Cortex Moutan dichloromethane fraction was carried out. 2,5-dihydroxy-4-methylacetophenone, 2,5-dihydroxy-4-methyoxyacetophenone and 3-hydroxy-4-methoxyacetophenone were isolated, and their structures and identities were confirmed by analysis with mass spectrometry and proton NMR. They had proved to have inhibitory effects in BBMV assay, 2,5-dihydroxy-4-methylacetophenone had the most effective inhibitory effect. Together with paeonol, they all belong to acetophenones and showed anti-diabetic effects, which was not published before.

Only paeonol (200 and 400mg/kg) showed positive effects on OGTT by both acute (1 day) and chronic (8 days) treatment in animal tests. Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis mixture (1:1:1), Cortex Phellodendri, Rhizoma Corptidis, Radix Ophiopogonis and CM-D3 (2,5-dihydroxy-4-methylacetophenone) did not have any anti-hyperglycemic effect.

6.3 Result Interpretation

6.3.1 Result Interpretation of *In Vitro* Studies

In Chinese medical theory, formation of diabetes is mainly due to three reasons: 1) deficiency of *yin* inside stomach; 2) deficiency of *yin* inside kidney; and 3) deficiency of *yin* inside lung. Hence, the herbs commonly used to treat diabetes should restore *yin* and quench *heat* inside stomach, kidney and lung. Six herbs were chosen out from *in vitro* studies, they were Cortex Moutan, Rhizoma Alismatis, Rhizoma Smilacis Chinensis, Cortex Phellodendri, Rhizoma Copitidis and Radix Ophiopogonis. And among these six herbs, they all have similar properties that they are "slightly cold" or "cold" in nature and can enhance fluid formation (yin-nourishing effect).

Radix Ophiopogonis would be the most commonly used one. It belongs to the lung, stomach and heart meridian. It exerts the action by quenching *heat* in *qi* and lung, and help improving the feeling of thirsty, dry mouth (dehydration) and hungry. As it runs inside stomach meridian after application, it may explain that the inhibitory effect on BBMV glucose absorption is due to this phenomenon.

Cortex Moutan is the second most commonly used herb among the chosen herbs. It belongs to the kidney, liver and heart meridian. It exerts the action by removing pathogenic *heat* from blood, prevents insomnia, restlessness and reduces the feeling of thirsty. As the effect Cortex Moutan is carried through liver meridian, it maybe related to the strong inhibitory effect in gluconeogenesis in liver.

Rhizoma Alismatis belongs to the kidney and urinary bladder meridian, and used to quenching heat in lymphatic system. It could enhance yin restoration in kidney, hence improve the water homeostasis. This herb showed the most promising effect on Hs68 fibroblast glucose uptake enhancement, but it cannot find any relationship from the Chinese medical theory, it may imply that it is a new effect to be discovered.

For Cortex Phellodendri and Rhizoma Copitidis, they are both "cold" in nature, and they are rarely used in DM comparatively unless the patients have extreme *heat* in stomach Cortex Phellodendri belongs to large intestine, kidney, and urinary bladder meridian, while Rhizma Copitidis belongs to stomach, large intestine, liver and heart meridian. Their actions are both clearing of latent-heat and clearing damp. These two herbal extracts showed strongest effect in BBMV intestinal glucose absorption inhibition, which match that the actions of these two herbs are mostly in digestive system.

And Rhizoma Smilacia Chinensis is not a widely used traditional Chinese herb. It belongs to liver and kidney meridian. It can clear damp. The herbal water extract of this herb can inhibit the gluconeogenesis in liver, as Chinese medicine theory regards it as a liver meridian running herb.

There are some differences between our research findings and other reported data among the three potent herbs chosen using *in vitro* screening studies. For 3T3-LI glucose uptake study, it had been reported that crude extracts of Cortex Phellodendri and Radix Ophiopogonis could enhance uptake of 2-DOG by percentages of 142 (0.1mg/ml), 132 (1mg/ml), 24 (10mg/ml) and 489 (0.1mg/ml), 374 (1mg/ml), 344 (10mg/ml), respectively in non-insulin-stimulated cell. In insulin-stimulated cells, the percentage of 2-DOG uptake was measured and found to be 747 (0.1mg/ml), 523 (1mg/ml), 33 (10mg/ml) and 753 (0.1mg/ml), 740 (1mg/ml), and 421 (10mg/ml), respectively. That indicated that Radix Ophiopogonis increased the glucose uptake in basal and insulin-stimulated 3T3-L1 adipocytes (Hong *et al.*, 2000). However in our research result as shown, both Cortex Phellodendri and Radix Ophiopogonis did not stimulate glucose uptake into 3T3-L1 adipocytes in both insulin treated and non-insulin treated cells. The main reason for the contradict any results should be due to the short

herbal extract incubation time (30 minutes) in our study, while another report mentioned above used one hour for incubation.

6.3.2 Result Interpretation of Cortex Moutan Purification

It was proved that hexane fraction of Cortex Moutan had significant anti-hyperglycaemic effect in STZ rat model (Lau, 2004). That means there was active component(s) inside Cortex Moutan. According to this hypothesis, further fractionation was done.

BBMV assay was applied in the bioassay-guided fractionation, so to isolate active compound(s) stepwise and the most potent fraction was selected according to BBMV results.

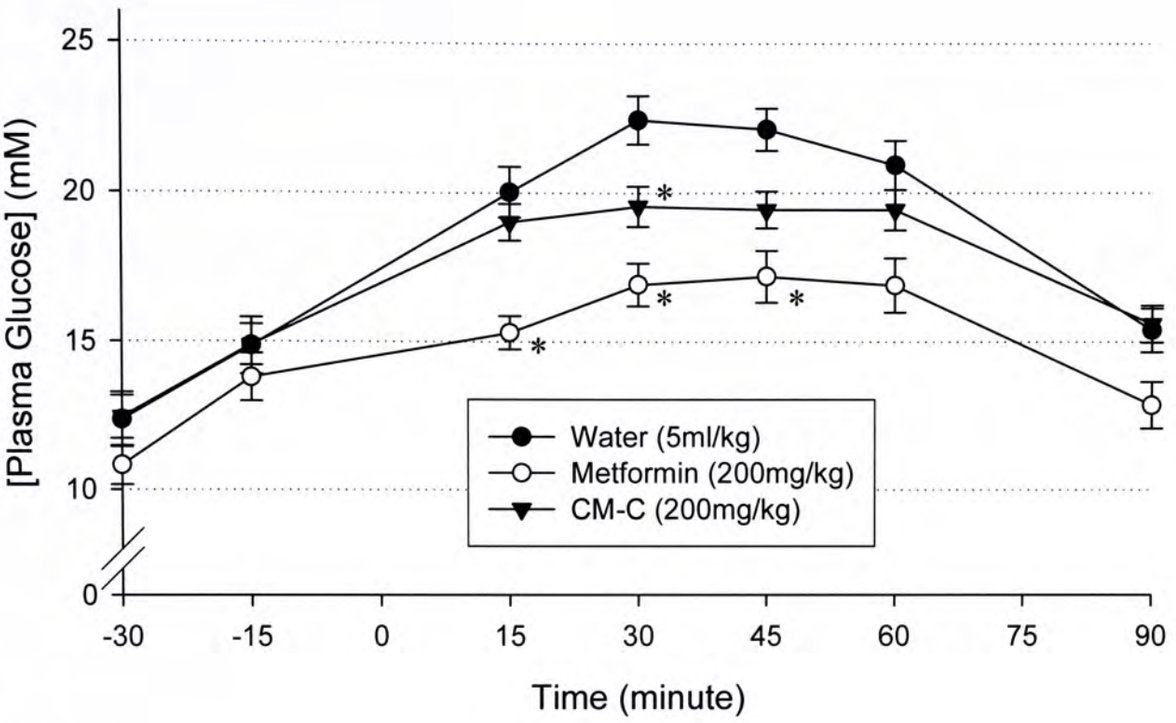
Further fractionation of Cortex Moutan was made from dichloromethane fraction (CM-D) rather than hexane fraction (CM-C). It was because the amount of suspected active component(s) inside CM-D fraction was shown to be more than that of CM-C in thin layer chromatogram (Fig. 4.4). Then five fractions namely CM-D1 to CM-D5 were purified.

The percentage yield CM-D3 purified was very low (around 0.13%). Although it is a known compound, it is not available commercially. Hence synthesis was needed in order to provide enough amount of CM-D3 for *in vivo* study.

Acetovanillone (apocynin) is a known compound inside Cortex Moutan. It is a NADPH inhibitor (Riganti *et al.*, 2005), and had a potent anti-inflammatory activity was found in rats with experimentally induced local or systemic inflammation (Hart and Simons, 1992). Under the purification condition in this project, it was not purified from hexane or dichloromethane fractions. As it also belongs to acetophenone family, it was

purchased and studied together with other acetophenones purified, so the actions between each structurally similar compound can be made.

(a)



(b)

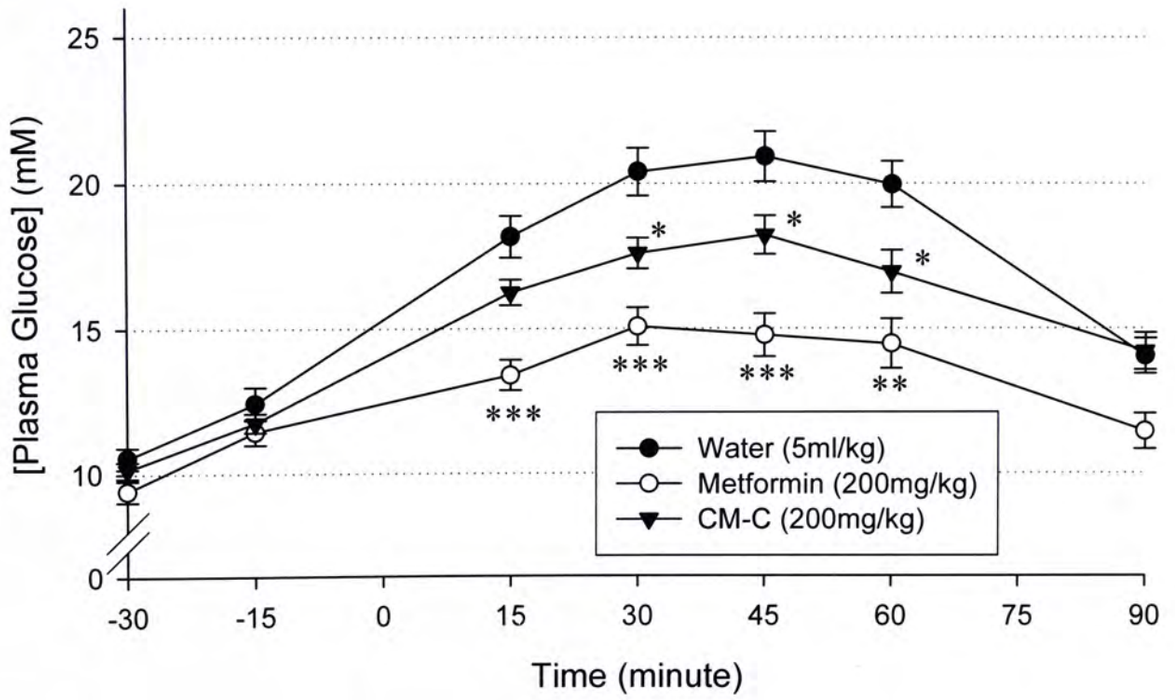


Fig. 6.1 Oral glucose tolerance test with Cortex Moutan fraction CM-C. (a) Day 1 acute experiment and (b) Day 8 chronic experiment. Metformin (200mg/kg) and CM-C (200mg/kg) showed significant anti-hyperglycaemic effects on both days. Results expressed as mean ± SEM, n = 18 to 22. Significantly different from water control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.3.3 Result Interpretation of *In Vivo* Studies

In other reseach studies about *in vivo* assays, Berberine, which is an active component of Rhizoma Coptidis, showed anti-hyperglycaemic in normal rats with i.p.glucose injection (Chen and Xie, 1986). And the polysaccharides isolated from Radix Ophiopogonis could significantly lower the blood glucose in alloxan-induced diabetic mice (Zhang and Wang, 1993) In our result as shown, there was no significant effect in anti-hyperglycaemia in OGTT with Rhizoma Coptidis nor Radix Ophiopogonis treatment rat groups. It might be due to two reasons: (1) the treatment used was only herbal water extract, hence the effect was not as strong as pure compounds; and (2) the dosages used were not high enough.

In vivo model showed that Cortex Moutan water extract (388mg/kg) had a trend to lower the blood glucose; for Rhizoma Alismatis and Rhizoma Smilacia Chinensis, although they exerted no effect in STZ model, but they show significant anti-diabetic effect *in vitro* (Lau, 2004), hence they were brought to synergistic effect study. But there was no synergistic effect showed in this study under ratio 1:1:1. This might be due to the mixture was made by mixing equal amount of individual herbal extract rather than extracted the raw herbs together.

For the acetophenones, from the *in vivo* results showed only paeonol exerted anti-hyperglyaemic effect in STZ rat model but CM-D3 and acetovanillone did not. There are several reasons leading to this result:

1. Structure-activity relationship

The structures of the above three compounds have high degree of similarity (Fig 6.2). When considering also CM-D4, CM-D5 and sodium paeonol sulfonate, we could make some hypothesis to explain the activity of paeonol. First, there should be a methoxy group at C4 position, a hydroxyl group at C2, and also with no

functional group on C5 position. For CM-D3, it had strong inhibitory effect in BBMV assay, but it did not have any effect in STZ rat model upon 1 day acute treatment. It did not have methoxy group in C4 but a methyl group instead; and there was a hydroxyl group on C5 position. These might be the crucial factors which affected its activity in animal model. For acetovanillone, the reasons were almost the same: it did not have methoxy group at C4 and had an empty C2 position.

2. Metabolism of Cortex Moutan active components in rats (Gjertsen *et al.*, 1988;Yasuda *et al.*, 1999)

There are some research about metabolism of paeonol and acetovanillone inside rat model. Each of the compounds was orally administrated to rats of dosage of 1mmol/kg, and the amount of the acetophenones and their derivatives were investigated in the excretory product. Paeonol was metabolized more extensively and larger amounts of demethylated (resacetophenone) and hydroxylated (mainly 2,5-dihydroxy-4-methoxyacetophenone CM-D4 and a trihydroxyacetophenone) metabolites were excreted. Paeonol did not find to have ketone reduction. Acetovanillone was rapidly excreted in the urine, mainly unchanged but also as the demethylated compound and three ring-hydroxylated metabolites. Minor additional metabolic pathways produced the para-methoxy derivatives, acetoisovanillone etc.. Ketone reduction occurred to form dimethoxy-hydroxy derivative and 1-phenylethanol derivatives. The metabolites were excreted mainly as glucuronide and/or sulphate conjugates. And the unchanged paeonol and acetovanillone mostly excreted in urine after 48 hours of application with percentage of 97% and 61% respectively. These facts reveal that paeonol as its structure could be altered during digestion, which meant it was being used and it could exert the effect to inhibit glucose absorption in digestive system; while acetovanillone, it was mostly excreted

out of the rat body, it could not exert effect on digestive system to stop glucose absorption. These facts might help to explain the negative result on OGTT of acetovanillone.

This project confirms the anti-diabteic effect of paeonol which was previously studied by another student. For the other three acetophenoic compounds isolated from Cortex Moutan, they are first reported to have inhibitory effect in BBMV assay. For CM-D3, the anti-diabetic effect in animal model needs to be further studied, as the sample number was not large enough to conclude the effect.

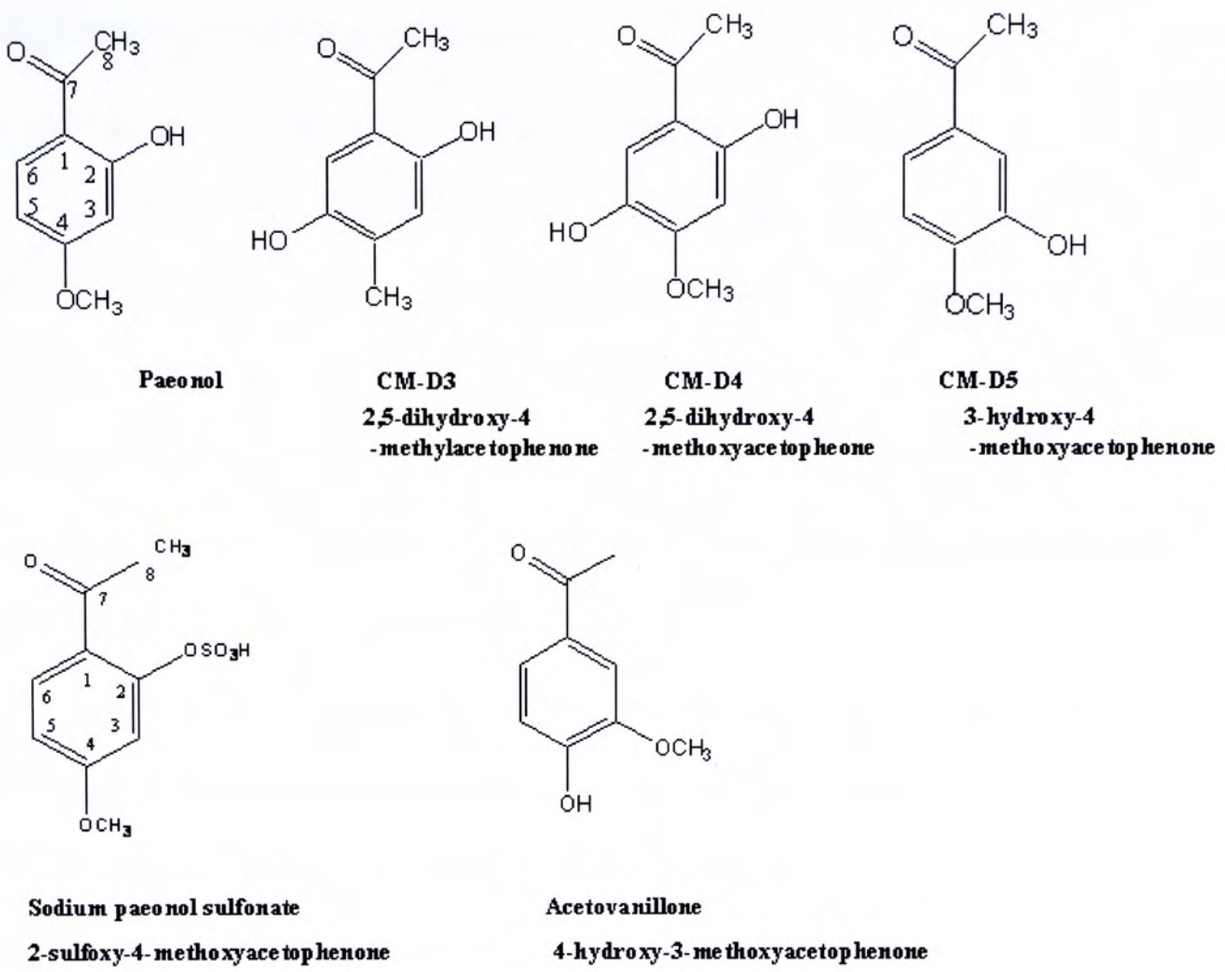


Fig. 6.2 Structures of acetophenones.

6.4 Limitations and Improvements

In Chinese medical theory, effect of the herb is not only focus on specific mechanism or organ, but also improving the symptoms of a disease through several mechanisms. Each *in vitro* model used in this project was only representing one specific mechanism. Although four *in vitro* assays together can give us a relatively complete screening system on glucose homeostasis, some hidden mechanisms responding to type 2 DM formation were still not yet disclosed and studied. Hence, *in vitro* assays may not be the best method to study the effect of the herbs.

But at this stage, *in vitro* models still play an important role in Chinese medical research. Since traditional Chinese medicine is an experience of ancient people and herbalists, it lacks scientific data to support its effect and application rationale. By using scientific research techniques, we can support the method of traditional usage of the herbs. Also, this screening system with different cell models provided a good method for experiment in large scale. Also, *in vitro* studies were much cheaper and providing faster results compared with *in vivo* study. *In vitro* study could be a very first step in narrowing down the herb list before further investigation in purification and animal model.

There are some limitations on both *in vitro* and *in vivo* assays. In BBMV assay, as a very small amount of glucose incubation reagent was needed, and hence, it is very sensitive to glucose. Any extra glucose (e.g. glucose presence inside herbal water extract) would affect the accuracy and gave false positive results. Until now, there is no effective way to remove glucose inside herbal water extract without remaining many other components. Hence non-polar solvents such as hexane and dichloromethane were used as extraction medium as glucose could not dissolve in them.

In 3T3-L1 glucose uptake assay, the degree of differentiation of 3T3-L1 cells varied between each experiment. It was observed that the degree of differentiation was greatly related to number of passage in cell culture. The cells would grow and differentiate better under smaller passage numbers, and so it is better to keep the passage number not higher then 6^{th}.

The diabetic stage of the *in vivo* model used in this project was not steady enough, the blood glucose level increased as the age of rat increased (serious complications such as retinopathy would occur). As a result, chronic treatment could not be studied in this model. Another *in vivo* model such as genetic diabetes mice should be employed as they have much more steady blood glucose level.

The theory of traditional Chinese medicine does not focus on specific illness, but to improve the *yin* and *yang* balance, and also *qi* restoration on whole body, so as to achieve the recovery of special organ, and maintain balance of different part of body. The traditional Chinese herbs usually exert a slow action in improving symptoms of an illness, rather than giving an acute response (Lei, 2003). Moreover, formulae with more than one herb are commonly used in treatment to give a synergistic effect rather than having one single herb as the effect of one herb is not strong enough. Interactions between each herb in formulae may also give rise to new compounds, and also neutralize the toxicity of some herbs (Zhang and Wumanjiang, 2003).

The *in vitro* studies used in this project served as screening systems of potential anti-diabetic traditional Chinese herbs. But the incubation time of herbal extract in each assay was short (several seconds to 3 hours), it could not show the effect of chronic treatment of the herbs.

The three herbs which showed the most potent effect *in vitro* did not show any anti-diabetic effect *in vivo*. The contradicted results might be due to the chronic effect of

TCM. As mentioned before, TCM usually emphasizes on improving the interior *yin* and *yang* balance, it might not exert a spontaneous action on lowering of blood glucose level immediately after administration as fast as that of western medicine; on the contrary it might slowly improve the glucose homeostasis by acting on related target cells such as pancreatic β-cells, liver and adipocytes etc., and this might need weeks to months to show the effect. For improvement, long-term herbal extract administration could be done in any animal model, and the chronic effect of the extracts could be studied.

The use of TCM is commonly done by first boiling the herb with water, and then followed by drinking the extract. Most of the studies in this project used herbal water extracts, but not for the BBMV assays. In the BBMV assay, herbal organic (hexane and dichloromethane) extracts were used, and we found that the extracts also give effective anti-diabetic effects. It reveals that some active components are water soluble, but some are also water insoluble. The use of organic extract allows those water insoluble components to be studied.

6.5 Future Directions

After purification of other active components from Cortex Moutan (2,5-dihydroxy-4-methylacetophenone, 2,5-dihydroxy-4-methyoxyacetophenone and 3-hydroxy-4-methoxyacetophenone), further studies are needed to find out the structure-activity relationship between each acetophenone (including paeonol and acetovanillone) as we could see from the *in vitro* results that different functional groups on different positions of acetophenones could affect their activities. To achieve the purpose, other similar structure compounds could be purchased or modify the structures by chemists, and the anti-diabetic mechanism of these compounds could be studied in animal model. This experiment could investigate the anti-diabetic activities inside animal, and the final aim

is to bring the effective compound(s) to clinical trial.

Paeonol showed positive results in both *in vitro* and *in vivo* assays, and it has potential to be leading compound anti-diabetic drug development. The disadvantages of developing paeonol are: 1) Paeonol showed low acute toxicity after i.v. injection, i.p. injection and orally feed of dosage 196mg/ml, 781mg/ml and 3430mg/ml respectively into mice, the toxicity observed were inhibitory effect in central nervous system, decreasing in body temperature etc.; 2) The blood glucose lowering effect of paeonol was only about 50% compared with that of metformin (a commonly used western oral medication), and it did not give a strong reason to support its development. However, there are some advantages of the developing paeonol: 1) Metformin has many side-effects, such as diarrhoea, nausea, lactic acidosis etc., paeonol, which is purified from commonly used nature plant and widely used by Chinese people, is more safe to use and proved to have less side-effects under normal human dosage according to pharmacopoeia; 2) The action of metformin is to inhibit the gluconeogenesis and also improve the insulin sensitivity in peripheral tissues, while paeonol exerts its effect on more up-stream mechanism by inhibiting glucose uptake from intestine, acts as the first barrier to lowering glucose content inside the patient's body. Hence, it can be used as a leading compound of further drug investigation by lowering its toxicity and increasing activity by modifying its structure

The future investigation on the anti-diabetic effect of paeonol can be focusing on synergistic effect of metformin and paeonol. As metformin is usually administrated together with sulfonylureas, which reduce the blood glucose level after meal by two different mechanisms, hence it can be a possible aspect to study their synergistic effect, and the supplementary effect of paeonol on western drugs available nowadays.

Purification of active components from Radix Ophiopogonis is also possible.

Although it did not significantly improve oral glucose tolerance, it gave a high degree of intestinal glucose absorption suppression in both hexane and dichloromethane fractions. The negative result in OGTT might be due to a high glucose content in this herb (the water extract of Radix Ophiopogonis is sweet in taste and very sticky in texture, and it was suspected to have high glucose content). To confirm this hypothesis, the glucose content of this herb should be investigated first.

Other mode of action in regulating blood glucose level could be investigated, such as protection and restoration of pancreatic β-cell function (Cabrera-Valladares *et al.*, 1999) and glucose uptake in skeletal muscle (Logie *et al.*, 2005). In type 2 diabetes, patients have limited insulin secretion and insulin resistance (World Health Organization, 1999). Further study is needed on the enhancing effect of insulin secretion and improvement of insulin sensitivity.

The *in vivo* synergistic effect on Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis was studied in this project, but negative results were obtained. This study was done by mixing three herbal water extracts of the same amount and administrated to STZ-rats, so the *in vivo* result was from the effect of individual herb. Further studies could be done by extracting three herbs together with water, and there might be a chance to form new compounds with effective anti-diabetic effects.

Some other genetically diabetes animal models can be used instead of STZ diabetic rat model in terms of investigating anti-diabetic effect of herbs (Liang *et al.*, 2005):

1. Genetic diabetic mice (C57BL/KsJ-m+/+Lepr) [by ♀m+/+db crossing ♂m+/+db]

   +db/+db : black, obese, *m+/+db* : black, lean, *m+/m+* : grey, lean:

   Female *+db/+db* mice and their female lean littermates (C57BL/KsJ-$Lep^{+/?}$) with 6–8 weeks of age will be used. They are housed 10 mice per cage and given access to water and food *ad libitum*.

The *+db/+db* mice and age-matched heterozygous lean littermates (control group) will be each randomly divided into different treatment groups. Herbal treatments with dosage equivalent to human dosage were administered via oral gavage once per day for 30 days. Body weights of the mice will be measured on the first and every 7 days of dosing. On day 31, OGTT are performed after fasting the mice overnight (18 hours), a drop of whole blood samples will be collected from tails onto sample window of blood glucose electrode (Precision Q.I.D., MediSense, Abbott). The blood glucose level will be measured by blood glucose sensor (Precision Q.I.D., MediSense, Abbott) by inserting the electrode into the sensor. Blood glucose level of diabetic control mice is steady and the level is around 16mmol/L.

2. Femle Zucker diabetic fatty (ZDF) rats

   Female ZDF rats at 6 weeks of age (ZDF/Gmi-fa) are housed four rats per cage and given access to water and food *ad libitum*. To develop hyperglycaemia, these rats were fed with a diabetogenic diet for 3 weeks. After 3 weeks on the diabetogenic diet, only the hyperglycaemic rats (blood glucose > 300mg/dl) were selected for the studies, and these ZDF rats were maintained on the diabetogenic diet through the entire study period.

   The rats are each randomly divided into different treatment groups. Herbal treatments with dosage equivalent to human dosage were administered via oral gavage once per day for 1 day (acute treatment) and for 30 days (chronic treatment). Body weights of the rats will be measured on the first and every 7 days of dosing. On day 31, OGTT are performed after fasting the rats overnight (18 hours), blood samples will be collected from tails at 15, 30, 45, 60 and 90 minutes in heparinized tubes after glucose challenge. Blood plasma will be collected by centrifuging the

whole blood sample at 4000 rpm for 5 minutes. The plasma glucose level will be measured by glucose assay kit.

Finally, a new formula of treating diabetes can be made by the six chosen herbs as they all exerted their anti-diabetic effect on different mechanisms according to the *in vitro* results (Table 6.2). They are all commonly used herbs in traditional herbal formulae, and it is reasonable to make them into one formula after consulting to an herbalist. This formula can be brought to in vitro assays and also *in vivo* assay again to investigate the synergistic effect, or by several trials, the number of herbs can be narrowed down according to the results.

Herb Name	H4IIE	BBMV (CH_2Cl_2)	BBMV (Hexane)	Hs68	3T3-L1
Cortex Moutan	-73.60% (5) *	/	-80.00% **	+24.30% (0.01) *	+78.90% (-insulin) (0.01)
Rhizoma Alismatis	-22.60% (5)	/	-8.20% **	+109.30% (0.1) ***	+82.80%(-insulin) (1)
Rhizoma Smilacia Chinensis	-81.90% (5) *	/	-7.36%	+51.8% (0.01) *	+87.70% (-insulin) (0.1) *
Cortex Phellodendri	-81.55% (5) **	-23.50% *	-69.48% ***	+58.19% (0.1) *	+33.32% (+insulin) (10) +63.44 %(-insulin) (1)
Rhizoma Copitidis	-64.01% (1) **	-28.63% **	-58.13% ***	+58.19% (0.01)	-35.28% (+insulin) (1) * +79.03% (-insulin) (0.1)
Radix Ophiopogonis	+9.22% (0.2)	-93.10% ***	-98.13% ***	+50.45% (0.1)	-26.11% (+insulin) (0.01) -3.81% (-insulin) (0.1)

Table 6.2 Overall summary of *in vitro* studies of six chosen traditional Chinese herbs. The number blanketed represents the concentration in mg/ml aqueous extract, except in BBMV studies with hexane or dichloromethane extracts. Significantly different from control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.6 Conclusions

A study platform with various *in vitro* models was established for the screening of potential anti-diabetic agents with different modes of actions.

Although *in vitro* studies suggested potential anti-diabetic effects of Rhizoma Copitidis, Cortex Phellodendri and Radix Ophiopogonis, the effects were not shown in animal studies. Cortex Moutan, Rhizoma Smilacis Chinensis and Rhizoma Alismatis water extracts did not possess *in vivo* synergistic anti-diabetic effects.

Cortex Moutan hexane and dichloromethane fractions potently inhibited intestinal glucose absorption *in vitro*, and paeonol was responsible for part of the activity in both fractions, while other minor acetophenones were responsible for another part of the activities. The anti-hyperglycaemic effect of paeonol was further confirmed in this report. The structure-activity relationship of all purified components needed to be further studied. Their pharmacological and phytochemical properties, as well as the potential of being new anti-diabetic agents should be further explored.

Appendices

Appendix 1 Survey on the formulae of diabetes and diabetic foot ulcer (By Mr. Lau Tai Wai)

Amount of various component herbs in the formulae for diabetes mellitus and diabetic foot ulcer (unit: grams)

Remarks: The mark with "√" indicates the amount of usage is adjusted and considered by the practitioners.

糖尿病的方劑													
病程較短, 較年輕的病人, 熱盛為主, 兼有陰虛				糖尿病中期, 病人是 40-60 歲之間, 病程 2-10 年				糖尿病晚期, 患病超過 10 年, 年齡普遍超過 65 歲			其他		
瀉心湯合黃連阿膠雞子黃湯 (心火亢盛)	天麻鈎藤飲 知柏地黃湯 加減 (肝陽偏亢)	玉女煎 (胃火熾盛)	白虎湯 / 消渴方加減 (肺胃津傷)	補心丹 交泰丸 加減 (心腎不交)	當歸補血湯 一貫煎 加減 (心肝兩虛)	歸脾湯加減 (心肺兩虛)	生脈散加味 (心肺兩傷)	四君子湯合四神丸加味 (脾腎陽虛)	大小健中湯加減 (脾胃虛寒)	瓜蔞韭白半夏湯加味 (心陽不足)	知柏 (陰虛火旺) / 六味地黃丸 (腎陰不足)		
清心瀉火 滋養心腎	滋陰潛陽	清胃瀉火	生津止渴 清胃潤肺	交通心腎 滋陰清熱	養心補肝	補益心脾	益氣養陰 寧心潤肺	溫腎補脾	溫脾暖胃	溫通心陽	滋陰降火 滋陰補腎	藥材名	功能及功效
			30									天花粉	上中下消, 益氣養陰, 清熱, 養陰潤肺, 生津止渴

6		6	6	6								黃連	瀉胃火及心火,清瀉胃火,安神,清心養血
15	15	15	15	10	15	15	15					生地黃	上中下消,補益精氣,滋陰固腎,清熱,柔肝,養陰潤肺,生津止渴,養陰清熱,填補腎陰,益氣養陰,減少動脈硬化及微血管病變,養心肺之陰,降血糖,養血涼血
			25									藕汁	清熱,養陰潤肺,生津止渴
			√									葛根	益氣養陰
		10	10	10	10	10	10					麥冬	上消,清熱,養陰潤肺,生津止渴,養陰清熱,安心神,滋陰養血,養心肺之陰
		30	30									生石膏	上中消,清肺胃熱,清瀉胃火
										12		丹參	活血化瘀,宣通心脈,減少動脈硬化及微血管病,益心氣,補心血,溫中理氣止痛,降血糖
	10	10	10				10					知母	上中消,清熱,養陰潤肺,生津止渴,養陰清熱,益氣養陰,清肝經熱
		≦30		10							24	熟地黃	中下消,填補腎陰
		10										牛膝	引熱下行
	10	√										梔子	清肝經熱

	10										12	山萸肉	下消, 清熱, 生津柔肝
	10										12	山藥	中消, 扶正固本, 益氣健脾, 利濕, 引熱外瀉, 益肺, 健運脾胃, +黃蓍>降血糖
				10		10		10	15		9	茯苓 (去皮)	健脾利澀, 安心神, 健脾祛濕, 補益心氣及脾氣, 引熱外瀉,
	10										9	澤瀉	健脾利濕, 引熱外瀉
	10										9	牡丹皮	清肝經熱
					30		20					生黃蓍	
						10		炒 10				白朮	中消, 補益脾氣, 安心神, 健脾益氣, 勝濕
						20						黃蓍	下消, 益氣養陰, 補心肺氣, 減少動脈硬化及微血管病變, 健運脾胃, 固表, 扶正固本, 利水消腫, 托瘡生肌
				6			6	10				五味子	酸收固澀, 收斂肺氣
												桃仁	
												紅花	活血化瘀
					10							當歸	活血化瘀, 補肝血, 減少動脈硬化及微血管病變, 養血和血理氣

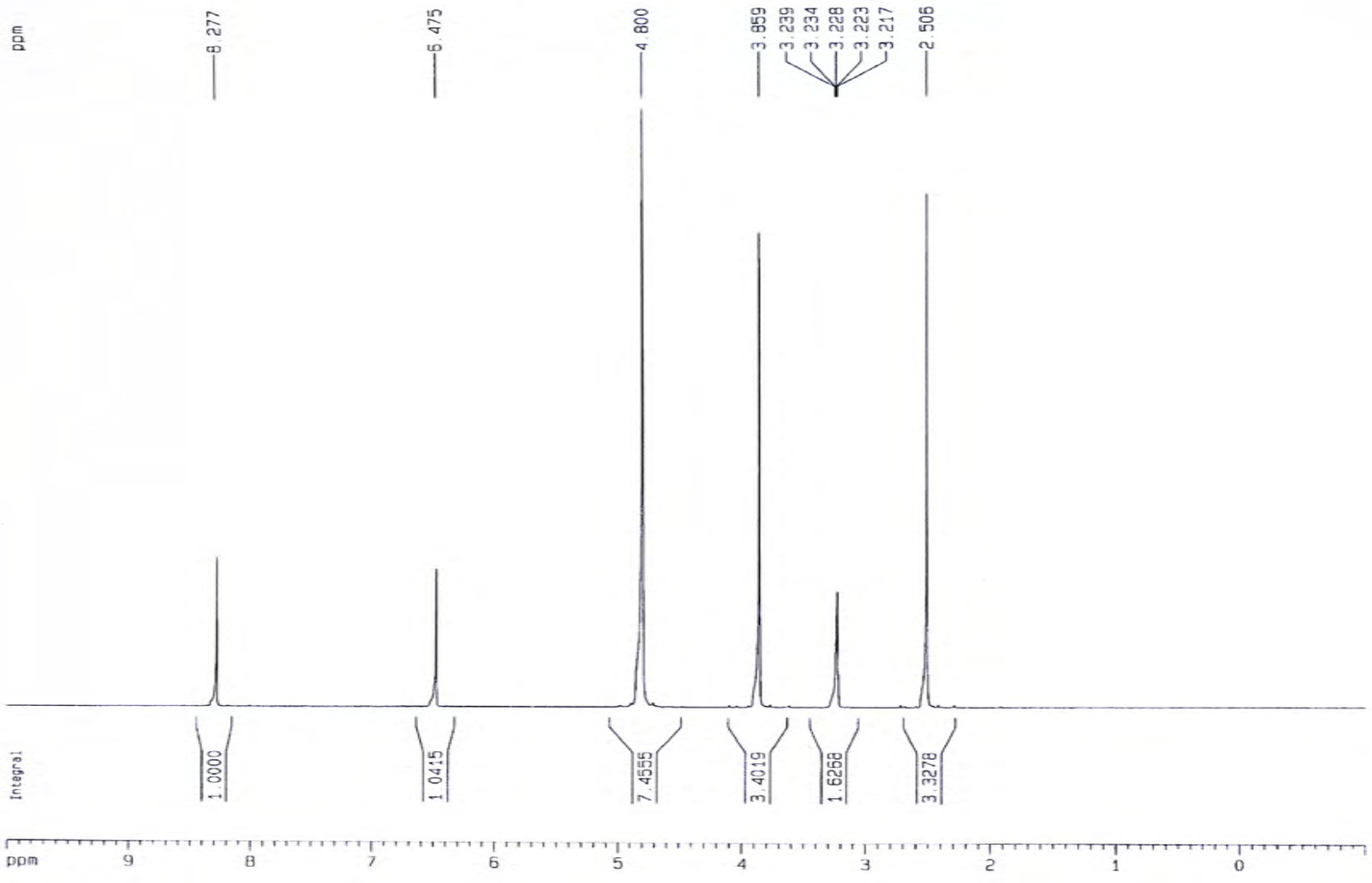
												川芎	活血化瘀, 減少動脈硬化及微血管病變, 降血糖
										10		赤芍	活血化瘀, 宣通心脈
				20		10	10					太子參	補心肺氣, 益氣養陰
						10						龍眼肉	
						6						木香	理氣健中
										10		郁金	活血化瘀, 宣通心脈
										10		法半夏	燥濕和中行氣
										10		韭白	通陽
					30							沙參	上消, 滋陰養血
												枳殼	
												柴胡	疏肝解鬱
			6					6	6			甘草	和中, 益胃護陰, 調和諸藥, 溫陽補血, 健脾益氣

												玄參	上下消, 生津止渴, 減少動脈硬化及微血管病變, 養陰, 降血糖
												銀花	清熱解毒, 消散癰腫
												蒲公英	治疔瘡癰瘍, 姓腫熱痛
												紫花地丁	治疔瘡, 無名腫毒
												白芷	
												鹿角膠	
												薑炭	
												白芥子	
												麻黃	
									6	6		桂枝	通陽, 溫陽補血
								12	12			黨參	健脾益氣, 健脾祛濕
10					10				10			白芍	健脾益氣, 滋肝陰, 養陰清熱, 溫陽補血, 養肝平肝, 柔肝緩急, 養血斂陰

												陳皮	
			10									天冬	清熱, 養陰潤肺, 生津止渴, 益氣養陰
					10							枸杞	下消, 養肝平肝
10			10									黃芩	清肺熱, 瀉胸膈之熱, +蒼朮>降血糖
			√									五味子	生津止渴, 滋腎水, 補肺氣, 補益心氣, 心血
			√									石斛	生津止渴, 養陰
			√									人參	益氣斂汗, 生津止渴, 益心氣, 補心血
6		6										淡竹葉	清心除煩利水, 養陰清熱
		√		12	12	12						炒棗仁	養陰安神
		√		12			12					柏子仁	養陰安神, 養心寧神
10												阿膠	滋陰養血, 清心, 安神
10	10											黃柏	瀉下焦實火和濕熱, 瀉腎火

6												木通	下利小腸引熱外泄
1 枚												雞子黃	養陰安神,清心安神
	6											天麻	平肝陽
	10											釣藤	平肝陽,鎮肝潛陽
	20											石決明	平肝陽
						7 枚			4 枚			大棗	安心神,溫陽補血
				2								肉桂	引火歸源,壯腎陽
								12				補骨脂	補腎氣,溫脾陽
								6				吳茱萸	溫中散寒
										10		枳實	燥濕和中行氣
								6				肉豆寇	溫腎暖脾
									6			幹薑	溫中散寒

									3			花椒	溫中散寒
									6			蒼朮	健脾祛濕, 降血糖, 清肺熱, 化濕
										15		金瓜蔞	開胸化痰
												菝契	祛風利濕, 解毒消癰
												制大黃	瀉熱通腑, 滌蕩積熱濕滯
												野菊花	癰腫疔毒
												敗醬草	
												紫背天葵子	攻癰疽, 排膿定痛
												桔梗	
												雞內金	健運脾胃

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PROCNO                    1

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Time                  9.50
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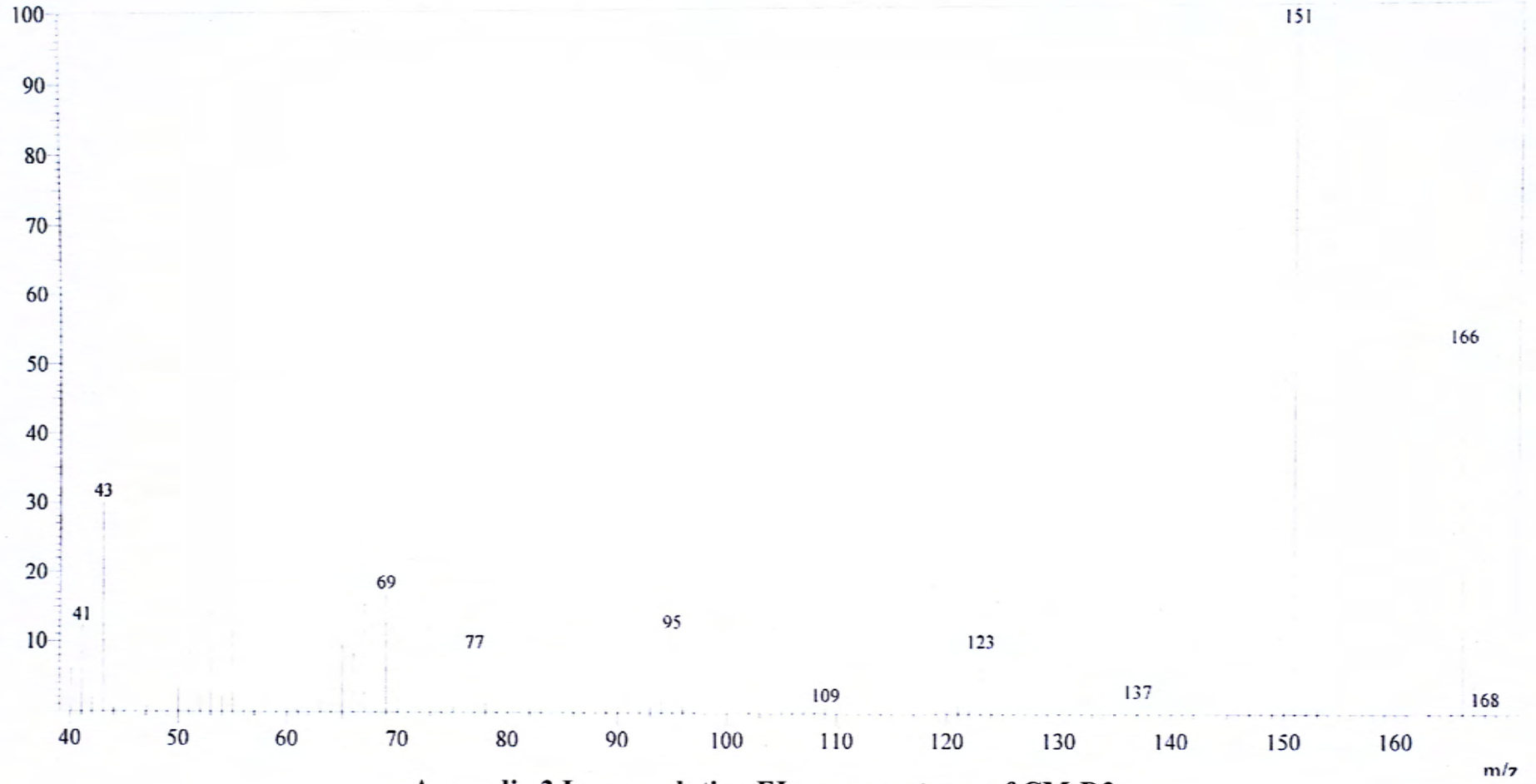
PULPROG                zg
TD                  32768
SOLVENT             CDCl3
NS                       16
DS                        0
SWH                5209.690 Hz
FIDRES             0.158987 Hz
AQ                 3.1449587 sec
RG                       64
DW                  95.975 use
DE                  137.11 use
TE                   297.2 K
D1                1.00000000 sec
MCREST            0.00000000 sec
MCWRK             0.01500000 sec

======== CHANNEL f1 =======
NUC1                     1H
P1                   5.00 use
PL1                 -2.00 dB
SFO1          300.1330580 MHz

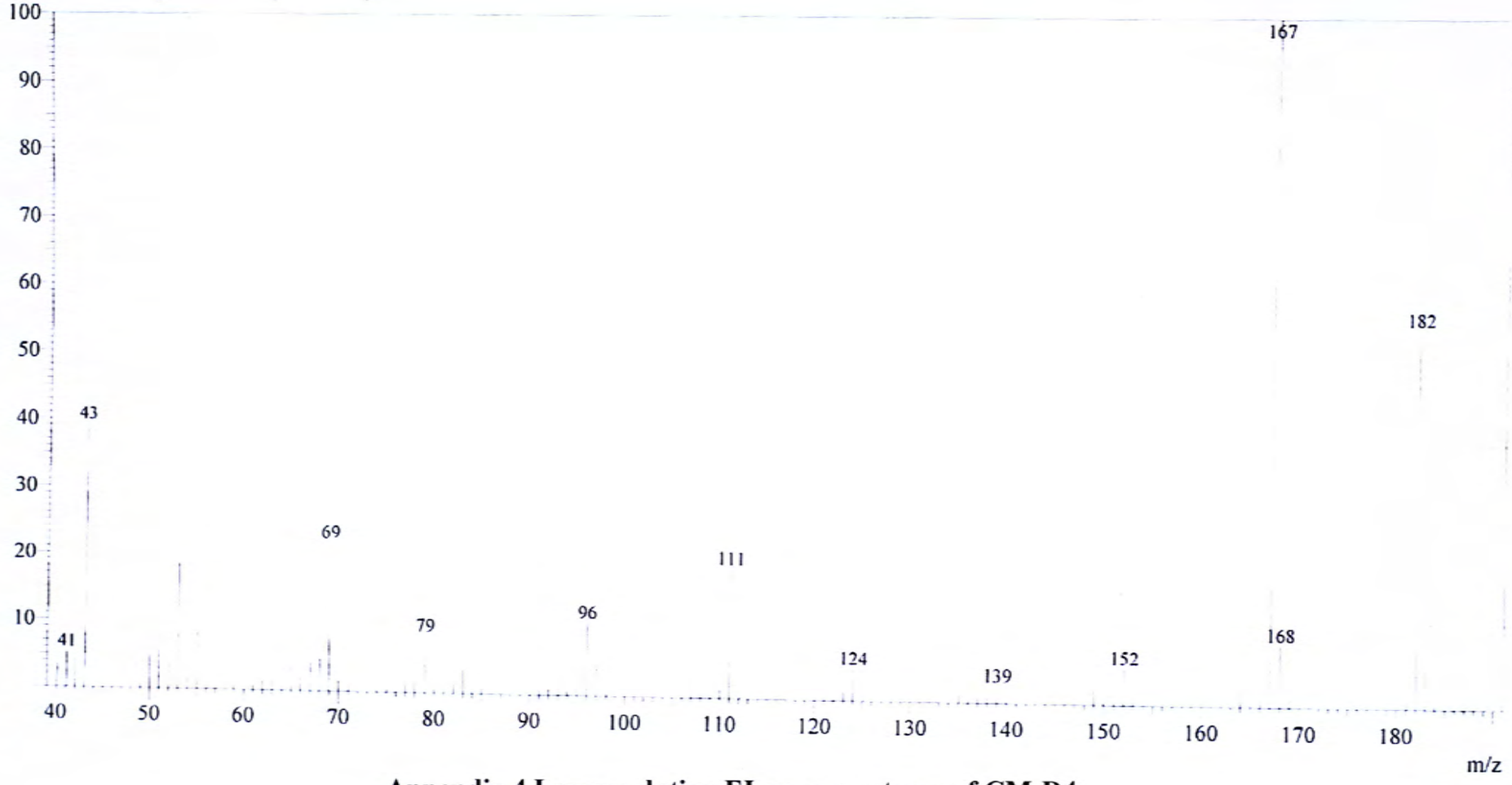
F2 - Processing parameters
SI                    32768
SF          300.1316524 MHz
WDW                      EM
SSB                       0
LB                   0.30 Hz
GB                        0
PC                   1.00

1D NMR plot parameters
CX                  22.00 cm
CY                  12.00 cm
F1P                10.000 ppm
F1                3001.32 Hz
F2P                -1.000 ppm
F2                -300.13 Hz
PPMCM             0.50000 ppm
HZCM            150.06583 Hz/
```

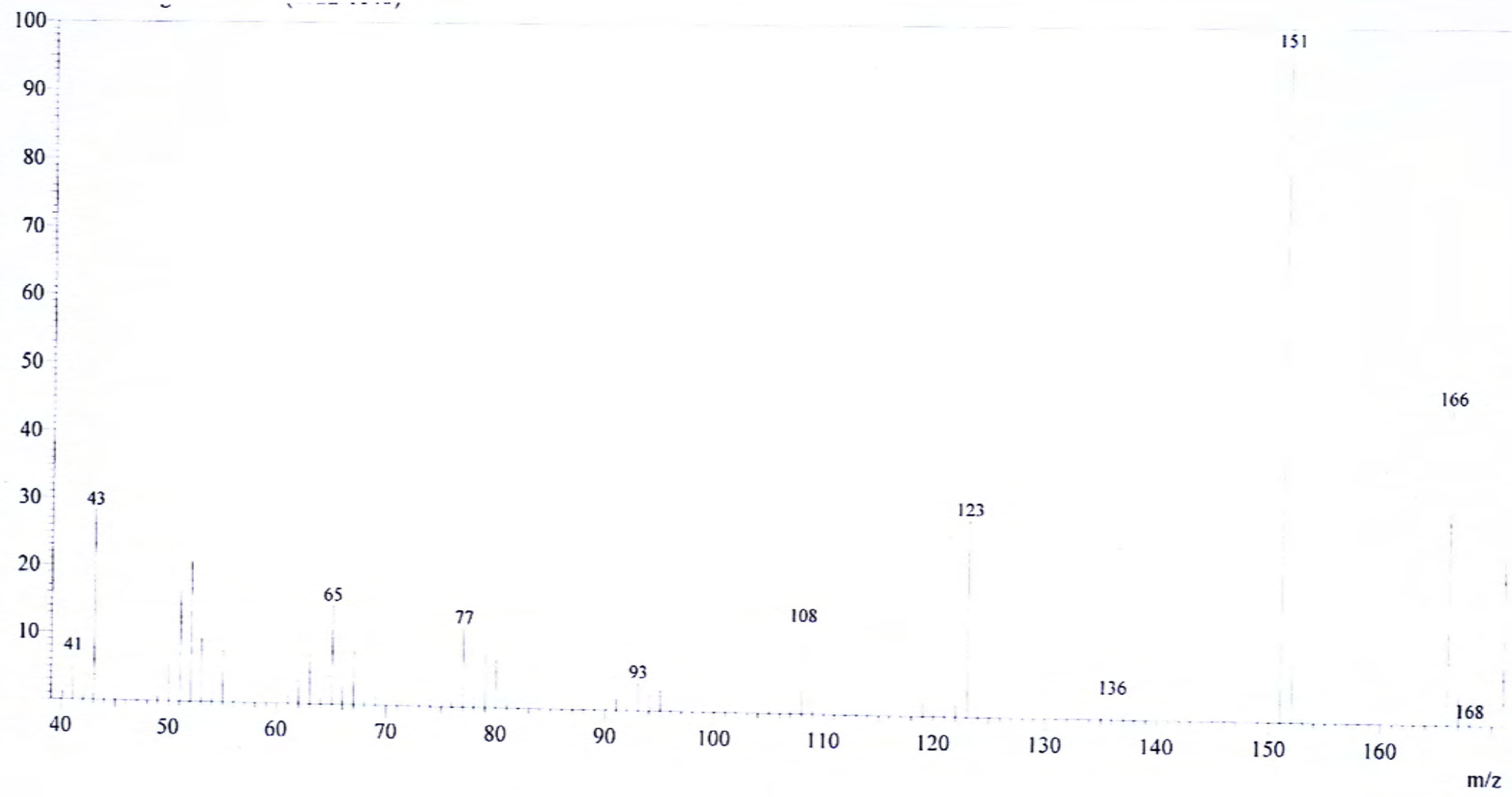
Appendix 2 ^{1}H-NMR spectrometry of CM-D1s



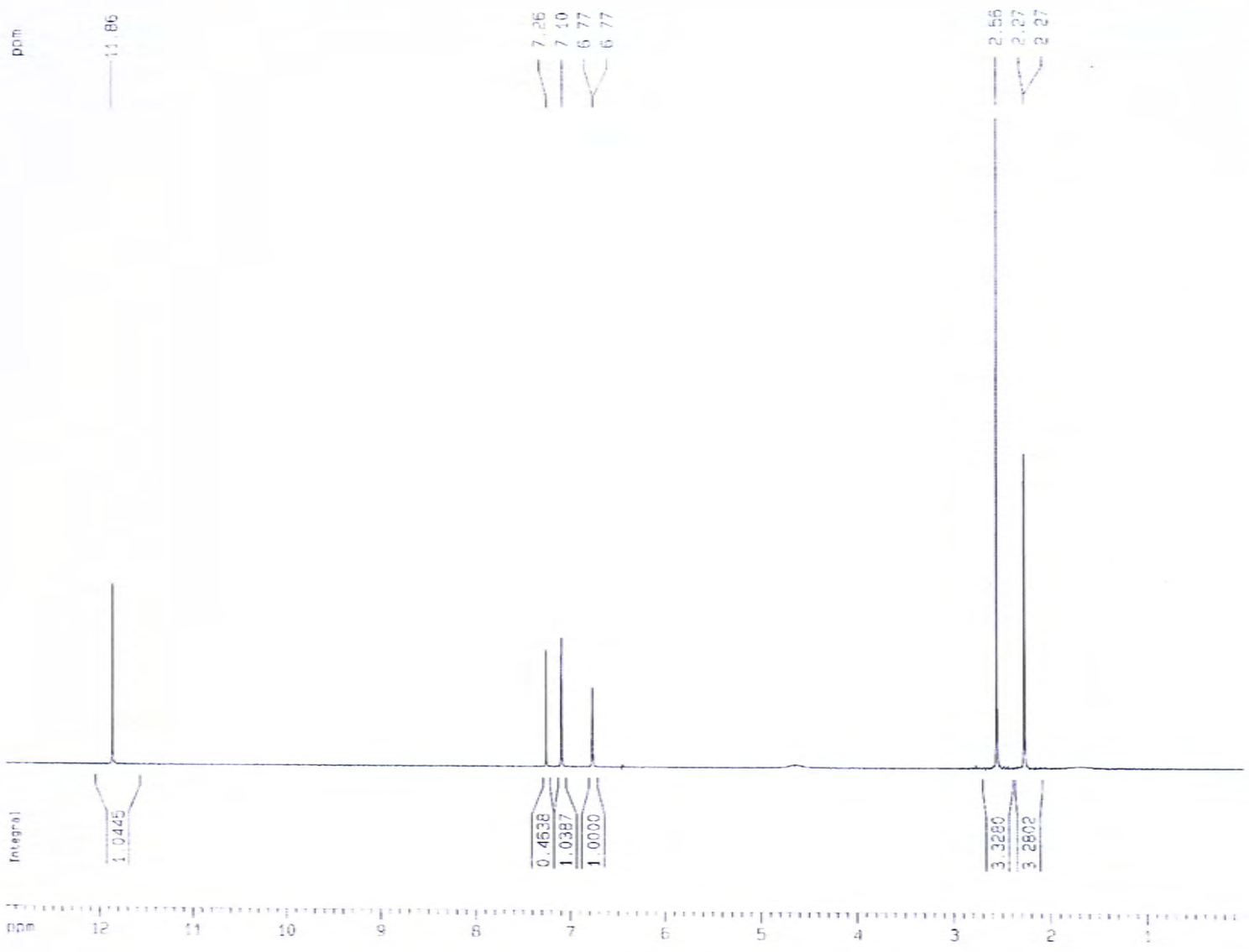
Appendix 3 Low resolution EI mass spectrum of CM-D3



Appendix 4 Low resolution EI mass spectrum of CM-D4



Appendix 5 Low resolution EI mass spectrum of CM-D6

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Current Data Parameters
NAME                  mdp-3s
EXPNO                      1
PROCNO                     1

F2 - Acquisition Parameters
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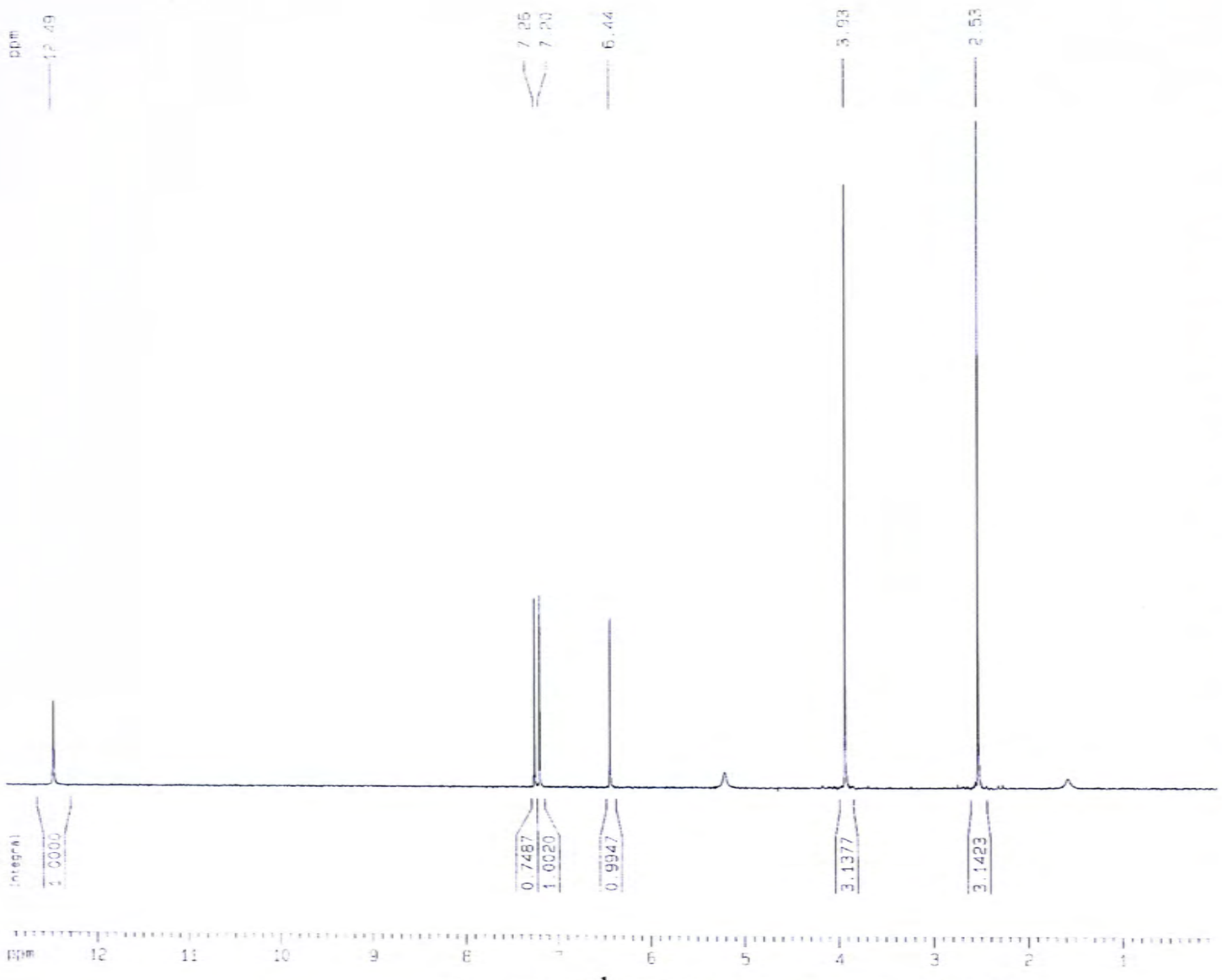
Time                  10.22
INSTRUM             dpx300
PROBHD   5 mm BBO BB-1H
PULPROG                zg
TD                    32768
SOLVENT               CDCl3
NS                        8
DS                        0
SWH                5185.377 Hz
FIDRES            0.158245 Hz
AQ                3.1597044 sec
RG                       10
DW                 96.425 usec
DE                 137.75 usec
TE                  298.2 K
D1                1.00000000 sec
MCREST            0.00000000 sec
MCWRK             0.01500000 sec

======== CHANNEL f1 ========
NUC1                      1H
P1                    3.00 usec
PL1                  -2.00 dB
SFO1           300.1305185 MHz

F2 - Processing parameters
SI                        32768
SF             300.1265804 MHz
WDW                       EM
SSB                       0
LB                    0.30 Hz
GB                        0
PC                    1.00

1D NMR plot parameters
CX                   22.00 cm
CY                   12.00 cm
F1P                 13.000 ppm
F1                 3901.67 Hz
F2P                 0.000 ppm
F2                    0.00 Hz
PPMCM              0.59091 ppm/cm
HZCM              177.34869 Hz/cm
```

Appendix 6 ^{1}H-NMR spectrometry of CM-D3




```
Current Data Parameters
NAME                  mdp-4s
EXPNO                      1
PROCNO                     1

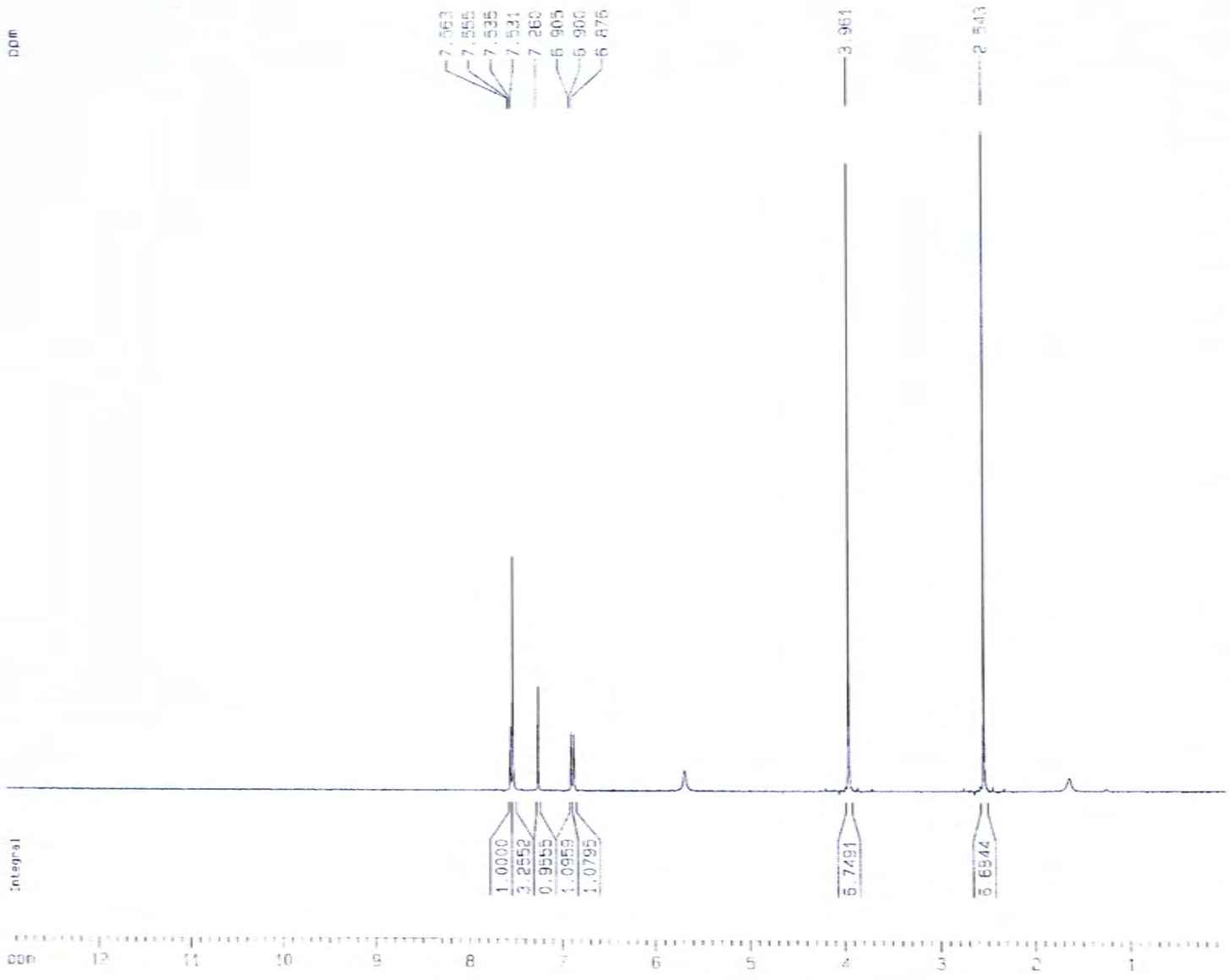
F2 - Acquisition Parameters
Date_              20050218
Time                   10.31
INSTRUM              dpx300
PROBHD   5 mm BBO BB-1H
PULPROG                zg
TD                     32768
SOLVENT               CDCl3
NS                         8
DS                         0
SWH                5185.377 Hz
FIDRES             0.158245 Hz
AQ                 3.1597044 sec
RG                        10
DW                   96.425 usec
DE                   137.75 usec
TE                    299.2 K
D1                 1.00000000 sec
MCREST             0.00000000 sec
MCWRK              0.01500000 sec

======== CHANNEL f1 ========
NUC1                      1H
P1                    3.00 usec
PL1                  -2.00 dB
SFO1           300.1305185 MHz

F2 - Processing parameters
SI                     32768
SF             300.1285804 MHz
WDW                       EM
SSB                        0
LB                    0.30 Hz
GB                         0
PC                    1.00

1D NMR plot parameters
CX                   22.00 cm
CY                   12.00 cm
F1P                  13.000 ppm
F1                 3901.67 Hz
F2P                  0.000 ppm
F2                   0.00 Hz
PPMCM              0.59091 ppm/cm
HZCM             177.34869 Hz/cm
```

Appendix 7 ^{1}H-NMR spectrometry of CM-D4




```
Current Data Parameters
NAME                  mdc-65
EXPNO                      1
PROCNO                     1

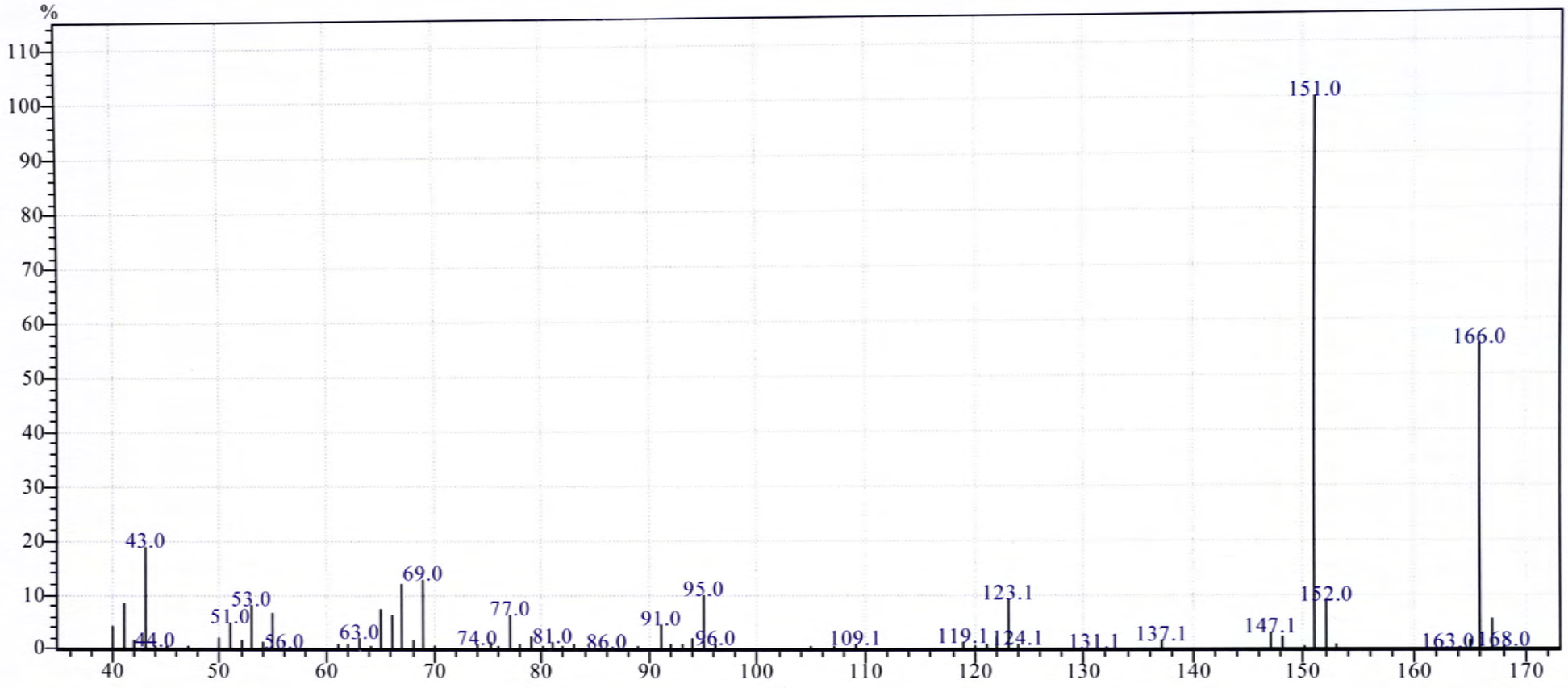
F2 - Acquisition Parameters
Date_              20050218
Time                   10.40
INSTRUM                spx300
PROBHD   5 mm BBO BB-1H
PULPROG                   zg
TD                     32768
SOLVENT                CDCl3
NS                         8
DS                         0
SWH               5185.377 Hz
FIDRES               0.158245 Hz
AQ                3.1597044 sec
RG                        10
DW                   96.425 usec
DE                   137.75 usec
TE                    299.2 K
D1              1.00000000 sec
MCREST          0.00000000 sec
MCWRK           0.01500000 sec

======== CHANNEL f1 ========
NUC1                      1H
P1                    3.00 usec
PL1                  -2.00 dB
SFO1           300.1305185 MHz

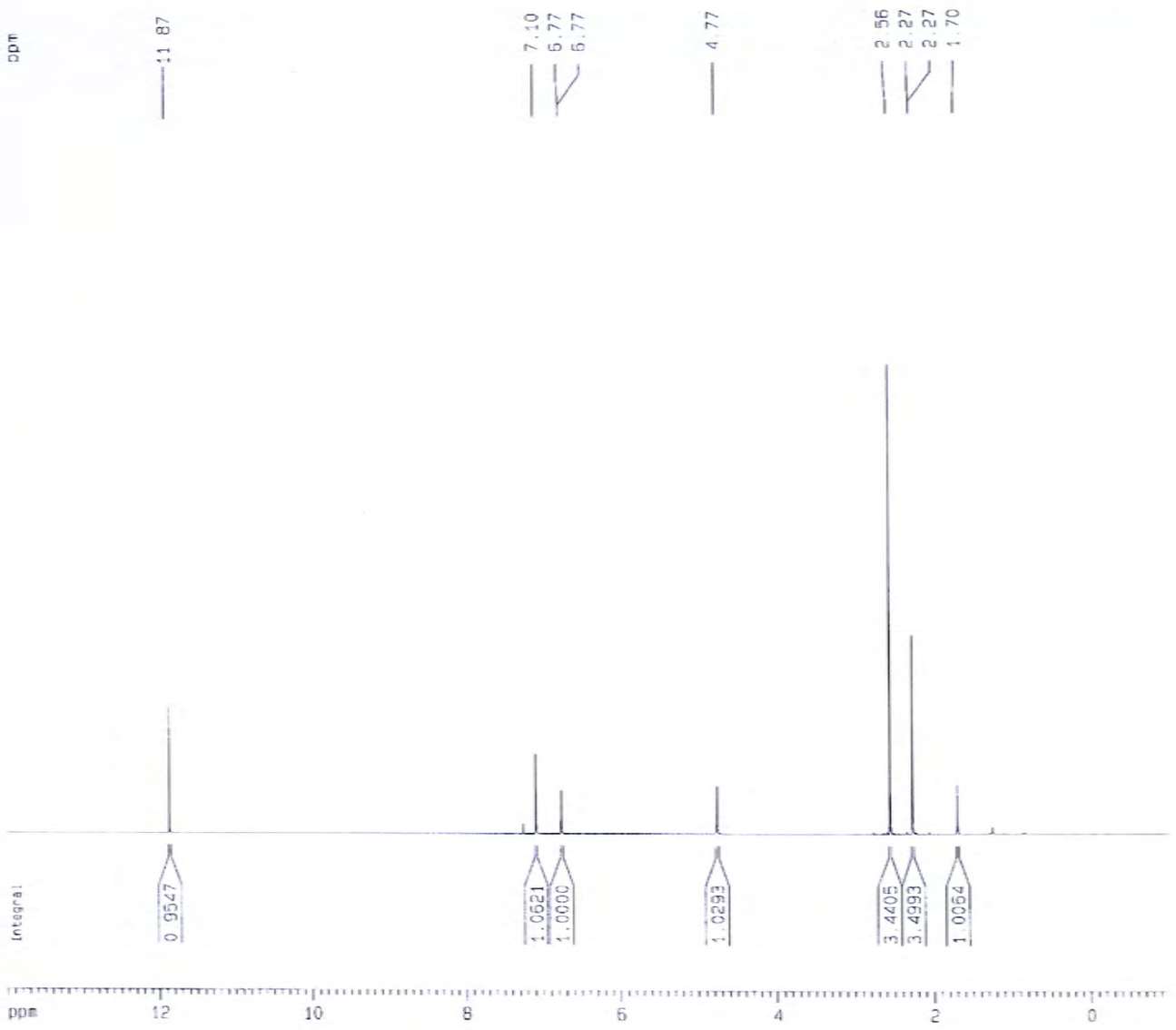
F2 - Processing parameters
SI                     32768
SF             300.1285802 MHz
WDW                       EM
SSB                        0
LB                    0.30 Hz
GB                         0
PC                    1.00

1D NMR plot parameters
CX                   22.00 cm
CY                   12.00 cm
F1P                 13.000 ppm
F1                 3901.67 Hz
F2P                 0.000 ppm
F2                    0.00 Hz
PPMCM              0.59091 ppm/cm
HZCM             177.34869 Hz/cm
```

Appendix 8 ^{1}H-NMR spectrometry of CM-D5



Appendix 9 Low resolution EI mass spectrum of CM-D3s

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Current Data Parameters
NAME                  ic#001
EXPNO                      1
PROCNO                     1

F2 - Acquisition Parameters
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Time                   10.07
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PROBHD   5 mm BBO BB-1H
PULPROG                   zg
TD                     32768
SOLVENT                CDCl3
NS                         8
DS                         0
SWH                8992.806 Hz
FIDRES             0.274439 Hz
AQ                 1.8219508 sec
RG                       256
DW                  55.600 usec
DE                   6.00 usec
TE                  297.2 K
D1                 1.00000000 sec
MCREST             0.00000000 sec
MCWRK              0.01500000 sec

======== CHANNEL f1 ========
NUC1                      1H
P1                    4.50 usec
PL1                  -2.00 dB
SF01             300.1312000 MHz

F2 - Processing parameters
SI                     32768
SF               300.1300063 MHz
WDW                       no
SSB                        0
LB                    0.00 Hz
GB                         0
PC                      1.00

1D NMR plot parameters
CX                    20.00 cm
CY                     8.00 cm
F1P                  14.000 ppm
F1                  4201.82 Hz
F2P                  -1.000 ppm
F2                  -300.13 Hz
PPMCM               0.75000 ppm/cm
HZCM              225.09750 Hz/cm
```

Appendix 10 ^{1}H-NMR spectrometry of CM-D3s

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